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ABSTRACT

**EFFECTS OF DIETARY CALCIUM AND FASTING
ON OSTEOCLAST RECESSION AND RECRUITMENT
IN CALCIUM DEFICIENT RATS**

by

Kenneth R. Wright

The osteoclast, a multinucleated giant cell, has as its role the resorption of bone matrix with consequent release of calcium. The osteoclast is thought to derive from a pluripotent stem cell which also gives rise to monocytes and macrophages. The development, activation, and inhibition of osteoclasts are influenced by a number of systemic and local factors. The release of these factors is influenced by changes in the dietary status of the animal. Calcium deficiency is known to stimulate the recruitment and activity of large numbers of osteoclasts. These osteoclasts can subsequently be inhibited by increasing dietary calcium intake. Disappearance of osteoclasts from the endosteum of long bones is complete after three days, but it is not known whether this phenomenon is gradual or whether there is a dietary calcium threshold that must be reached to cause complete osteoclast inhibition.

This study examines changes in osteoclast morphology in calcium deficient rats following calcium supplementation and fasting. In rats which had been given a calcium-deficient diet 7 days, 12 hours of *ad libitum* calcium supplementation caused changes in osteoclast morphology indicating a reduction in osteoclast activation. These morphological changes were reversed when calcium supplementation was followed by a 12-hour fast.

In another experiment, generalized nutritional deficiency (i.e. fasting) was compared with calcium deficiency. Forty-eight hours of fasting in normal rats caused a decrease in the osteoblast area fraction and an increase in the resting-cell area fraction; the osteoclast area fraction was unaffected. Forty-eight hours of calcium deficiency, however, increased the osteoclast area fraction and decreased the resting area fraction, while the osteoblast area fraction did not change. These results suggest that whereas fasting conserves plasma calcium by reducing the osteoblast area fraction and thus bone formation, calcium deficiency maintains plasma calcium by increasing osteoclastic bone resorption. A final experiment showed that osteoclasts that have been inhibited by 48 hours of calcium supplementation did not return to pre-inhibition levels of activity after 48 hours of calcium deficiency. It was concluded that osteoclasts are under dynamic control and that their responses to changes in dietary status are graded.

LOMA LINDA UNIVERSITY

Graduate School

**EFFECTS OF DIETARY CALCIUM AND FASTING
ON OSTEOCLAST RECESSION AND RECRUITMENT
IN CALCIUM DEFICIENT RATS**

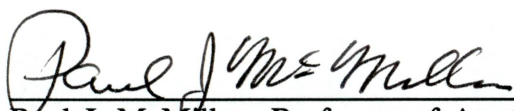
by

Kenneth R. Wright

**A Dissertation in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy in Anatomy**

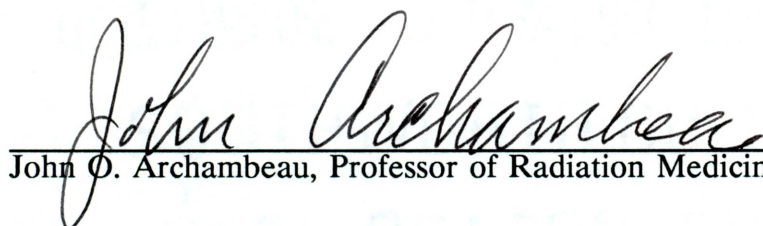
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LIST OF ABBREVIATIONS

1,25(OH) ₂ D ₃	1,25-dihydroxycholecalciferol
24R,25(OH) ₂ D ₃	24,25-dihydroxyvitamin D ₃
ANOVA	analysis of variance
BGP	bone gla protein; osteocalcin
bPTH	bovine parathyroid hormone
BSA	bovine serum albumin
Ca	calcium
CAM	chorio-allantoic membrane
cAMP	cyclic adenosine monophosphate
CI	osteoclast contact index
CSF	colony-stimulating factor
CT	calcitonin
D ₃	1,25-dihydroxycholecalciferol
EGF	epidermal growth factor
EDTA	ethylenediaminetetraacetic acid; (ethylenedinitrilo)-tetraacetic acid
EHDP	ethane-1-hydroxy-1,1-diphosphonate
FGF	fibroblast growth factor
GAG	glycosaminoglycans
G-CSF	granulocyte colon-stimulating factor
GH	growth hormone
GM-CSF	granulocyte-macrophage colony-stimulating factor
HHM	humoral hypercalcemia of malignancy
HLA	human lymphocyte antigens
hPTH	human parathyroid hormone
HX	hypophysectomized
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
II	osteoclast isolation index
IL	interleukin
i.v.	intravenous
LIF	leukemia inhibitory factor
M-CSF	macrophage colony-stimulating factor
MNC	multinucleated cell
MPS	mononuclear phagocytic system
OBAF	osteoblast area fraction
OCAF	osteoclast area fraction
OGF	osteoclast growth factor
op/op	osteopetrotic, a strain of mouse
ORSA	osteoclast resorption-stimulating activity
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PDGF	platelet-derived growth factor
PGE	prostaglandin E

PGI	prostaglandin I
P _i	inorganic phosphate
PTE	parathyroid extract
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
RAF	resting area fraction
RBI	ruffled border index
REM	removed index
sCT	salmon calcitonin
SD	standard deviation
SEM	standard error of the mean
SI	osteoclast separation index
T3	tri-iodothyronine
TGF	transforming growth factor
<i>tl</i>	toothless, a strain of rat
TNF	tissue necrosis factor
TraATPase	tartrate-resistant acid ATPase
TRAP	tartrate-resistant acid phosphatase

INTRODUCTION

In recent years, much attention has been given to the study of bone diseases such as Paget's Disease, osteopetrosis, and especially osteoporosis. With an increasing elderly population in America, the cost of caring for victims of osteoporosis is rapidly growing. Much effort is being spent pursuing new medications that will alter bone metabolism in such a way as to decrease bone loss. The loss of bone in osteoporosis is thought to result from an imbalance between bone resorption and bone formation. These two processes occur concurrently in normal individuals, but when resorption and formation are uncoupled, and resorption occurs to a greater extent than formation, the loss of bone mass results. To effectively control bone resorption, the mechanism by which it occurs must be understood. This involves a thorough knowledge of the biology of the bone-forming and bone-resorbing cells, the osteoblasts and the osteoclasts. This study focuses on the activity of the osteoclast.

Light microscopic examination of one μm sections of rat tibial diaphysis stained with toluidine blue reveals three cell types making up the endosteum, the layer of cells covering the bone surface that forms the boundary of the marrow cavity (see Appendix C). These endosteal cell types include the osteoblast, the bone lining cell or resting osteoblast, and the osteoclast. Osteoblasts and bone lining cells are believed to be essentially the same cell type at different levels of activation. The lining cell is a flattened, elongated cell, whereas the osteoblast is more cuboidal, with a distinct negative-staining Golgi region when stained with

toluidine blue. Both of these cell types are mononucleated. The osteoclast is normally the only multinucleated giant cell found in the endosteum.

The osteoclast is the cell responsible for bone resorption. The ultrastructure of the cell reveals machinery geared for high energy expenditure and osteolytic activity. The cytoplasm is especially rich in mitochondria, lysosomes, and vacuoles, and is amply stocked with Golgi and free ribosomes, and some rough endoplasmic reticulum (see Holtrop and King 1977). The active cell demonstrates a clear polarity. The plasma membrane adjacent to the bone surface is greatly folded forming a characteristic "**ruffled border**". Surrounding this ruffled border and forming a "sealing zone" is an area of cytoplasm devoid of organelles termed the "**clear zone**". An osteoclast that has been actively resorbing bone for a period of time sits in a concave excavation of bone commonly called a Howship's lacuna. Due to the degree of vacuolization and the sparser rough endoplasmic reticulum, the osteoclast's cytoplasm stains lighter, with a more "frothy" appearance, than either the osteoblast or the bone lining cell. At high magnification, the ruffled border and clear zone are visible with light microscopy. Thus, the osteoclast is easily distinguishable from the other endosteal cells at the light microscopic level.

Osteoclast Origin

Several theories have been proposed in an effort to explain the origin of the osteoclast. One theory, now abandoned, stated that the progenitor cell of bone osteoblasts and osteoclasts was a sessile connective tissue cell. A second

hypothesis for which supporting evidence is lacking proposed that osteoclasts derive from liberated osteocytes or chondrocytes. Current evidence, some of which will be presented below, suggests that the osteoclast progenitor is a hemopoietic stem cell, that it has a separate lineage from the osteoblast, and that it shares a common ancestor with the monocyte.

One of the earliest studies into the origin of the osteoclast was carried out by Fischman and Hay (1962) who used a regenerating newt limb model to show that osteoclasts are post-mitotic cells deriving from a leucocyte source. Tritiated thymidine injections were given 1 - 28 days after limb amputation, and animals were sacrificed 3 hours after thymidine injection. None of the osteoclasts in the tissue samples taken from these animals had labelled nuclei, indicating that no DNA synthesis had occurred within these cells. In a second experiment, tritiated thymidine was given at 5, 10 and 15 days after amputation, and while mesenchymal cells in the regenerating limb were found to be labelled, the osteoclasts never were, providing evidence that osteoclasts do not derive from mesenchymal cells. Finally, animals were labelled one day before amputation and followed for up to 28 days after amputation. It was found that labelled monocytes appeared in the regenerating limbs, with labelled osteoclasts appearing afterward. The authors concluded that osteoclasts derive from a blood-borne precursor cell, which they felt to be the monocyte.

Several groups provided evidence that osteoclasts and osteoblasts derived from separate lineages. Göthlin and Ericsson (1973) joined pairs of rats by

parabiosis, subjected one rat to whole-body irradiation to destroy hemopoietic stem cells, and shielded the other animal from the radiation. The shielded animal was given tritiated thymidine. In the shielded animal, many cell types including fibroblasts, chondroblasts, preosteoblasts and osteoblasts, as well as cells of the mononuclear phagocytic system (MPS), were labelled with the tritiated thymidine. However, in the irradiated animal only MPS cells were labelled showing that these cells had an extraskeletal origin, and that their origin was different from that of the osteoblast lineage. Miller et al. (1977) injected rats with ethane-1-hydroxy-1,1-diphosphonate (EHDP) over a period of six days, then gave $^3\text{[H]}$ -thymidine injections at 8-hour intervals for 40 hours. They found that EHDP caused an increase in the numbers of both osteoclasts and osteoclast nuclei, but the number of osteoprogenitor cells remained unchanged or decreased. While EHDP increased the numbers of osteoclasts, the rate of bone resorption was decreased. This led them to the conclusion that osteocytes, which would be released by osteoclastic bone resorption, could not be major contributors to the formation of osteoclasts, since few osteocytes were being released. Jotereau and LeDouarin (1978) grafted chick bone primordia onto Japanese quail chorio-allantoic membrane. Chondrocytes, osteocytes and osteoblasts that developed in the bone rudiment had chick-like nuclei, but myeloid and erythroid cells and osteoclasts had quail nuclei, again showing the extra-osteal origin of the osteoclast, and a separate origin for osteoclasts and osteoblasts. Kahn and Simmons (1975) had earlier used the quail/chick chimera

model to show that osteoclasts arise from the fusion of mononuclear hematogenous cells.

Yabe and Hanaoka (1985) later raised questions about the hemopoietic origin of the osteoclast. They also used quail-chick chimera, but arrived at different conclusions than Kahn and Simmons, and Jotereau and LeDouarin. In a first experiment, they grafted quail limb buds onto chick CAM, and they found that the osteoblasts and osteocytes which formed in the limb bud had quail-like nuclei, but the osteoclasts displayed either quail-type, chick-type or mixed-type nuclei. They next grafted either viable or devitalized pieces of Dunn osteosarcoma onto the CAM. In both cases, bone formation was induced, with osteoblasts and osteoclasts being formed. They reasoned that these cells couldn't have originated from devitalized osteosarcoma cells, so they must have come from the CAM. In their third experiment, they again used quail limb buds, but this time enclosed in a diffusion chamber, so that no cells could enter. The chambers containing the limb buds were grafted onto the CAM, and after 5 days incubation, PTH was administered, followed by 3 to 48 hours incubation time. Osteoclasts were found in those specimens that had received PTH and then went 48 hours before fixation. The authors concluded that the osteoclasts had to have originated from the mesenchymal cells (perichondrium) of the limb buds. Thus they assumed that mesenchymal cells give rise to osteoclasts.

The majority of studies, however, supported the idea of the myelogenic origin of the osteoclast. When Walker (1975) transplanted spleen or marrow cells from

normal mice to irradiated osteopetrotic mice, the osteopetrosis was cured. Marks and Schneider (1978) used this same model but by using the Ficoll-Hypaque method to separate various cells fractions from spleen or thymus cells, they proposed that it was the mononuclear cell fraction that gave origin to the osteoclast. Coccia et al. (1980) reported reversing human osteopetrosis by transplanting marrow from a male HLA identical sibling to a female patient. Evidence for the myelogenic origin of the osteoclast lay in the fact the osteoclasts in the cured female patient had fluorescent Y bodies upon staining with quinacrine, indicating that their precursors came from the marrow cells of the male donor. Sorell et al. reported similar results (1981). Ko and Bernard showed that the addition of mononuclear marrow cells to fetal mouse calvarial cultures was necessary to produce osteoclasts. Burger et al. (1982) found that osteoclasts appeared and ^{45}Ca was released in embryonic long bone cultures only when marrow or embryonic liver cells were added. Studies by Ibbotson et al. (1984), Fuller and Chambers (1987), Suda et al. (1992), Hattersley et al. (1991), and others (Takahashi et al. 1988b; Akatsu et al. 1989; Akatsu et al. 1989b; Akatsu et al. 1991) also confirmed the myelogenic origin of the osteoclast.

Although several studies showed that osteoclasts are not derived from macrophages (Fuller and Chambers 1987; Kurihara et al. 1990), research indicates a common origin for MPS cells (Athanasou et al. 1988; Hattersley et al. 1991).

Research is currently investigating stages of development of the osteoclast precursor into the mature osteoclast. An example is a study by Helfrich, Mieremet, and Thesingh (1989) showing that whereas the mature osteoclast stains positively for tartrate-resistant acid phosphatase (TRAP), early precursors are not TRAP+.

In summary, the current understanding of osteoclast origin is that the multinucleated osteoclast is formed by the fusion of mononuclear precursor cells which come from a different lineage than the osteoblast, and that they have a common myelogenic ancestor with the monocyte and macrophage.

Factors Involved in Osteoclast Recruitment, Activation or Inhibition

The regulation of osteoclast activity is a complex phenomenon involving both systemic and local factors such as hormones, cytokines and growth factors. A great amount of research is currently being done to elucidate the roles and interactions of these substances. The following is a brief summary of some of the findings having to do with factors influencing osteoclast activity.

Systemic Hormones

Parathyroid hormone (PTH)

Parathyroid hormone is an active agent in the regulation of blood calcium levels. Early studies of the hormone revealed seeming contradictions in its role in bone metabolism. Bauer, Aub and Albright (1928) found a reduction in long bone trabeculae when daily injections of parathyroid extract (PTE) were given to

rabbits and cats, and PTE given to growing kittens caused a diminution in long bone length but an increase in the number of trabeculae. Selye (1932) showed that high doses (overdoses) of PTE stimulated bone resorption (*osteitis fibrosa*) by osteoclasts, but that this phenomenon was followed by a disappearance of osteoclasts, with an increase in osteoblast numbers and bone formation, as was also later shown by Burrows (1938). A low dose of PTE brought about increased osteoblast numbers and bone formation, without the preceding bone resorption. Shelling, Asher, and Jackson (1933) confirmed this finding that a low dosage of PTH increases bone formation, while high doses cause resorption. This difference in the effects produced by PTH at low and high doses probably accounted for the results obtained by Tam et al. (1982), who found that daily injections of bovine PTH (bPTH) into rats increased the bone apposition rate and a net increase in trabecular bone (confirmed by Mosekilde et al. 1991), whereas continuous infusion of bPTH into rats caused an increase in both the formation and resorption areas with a net decrease in trabecular bone volume.

Barnicot (1945) used *grey-lethal* mice to show that high doses of PTE inhibited osteoblasts and increased bone loss, and that low doses of PTE stimulated osteoblastic activity, resulting in greater bone deposition. He later developed a system of transplanting parathyroid glands to pieces of parietal bone in mice, in order to determine the effects of the direct action of PTH on bone (Barnicot 1948). The bone pieces, with attached parathyroid glands, were grafted to a cerebral hemisphere of a litter-mate, and after 12-14 days, active osteoclastic

bone resorption resulted. Chang (1951) confirmed Barnicot's findings when, after transplanting parathyroid glands to parietal bones subperiosteally, he reported marked resorption of parietal bone around the grafts with minimal new bone deposition.

Later *in vitro* studies (Gaillard 1955; Raisz 1965) showed an increase in the number of osteoclasts and in bone resorption under the effects of PTE or PTH. Numerous studies have since shown increases in numbers of osteoclasts and osteoclast nuclei (Holtrop et al. 1979; Baron and Vignery 1981), as well as increases in osteoclastic activity as evidenced by changes in morphology (Holtrop et al. 1979; Miller, Bowman, and Myers 1984; Pandalai and Gay 1990) brought about by PTH. Data from Lorenzo et al. (1983) suggest that PTH acts on already-differentiated osteoclasts or osteoclast precursors, but does not induce DNA synthesis in undifferentiated osteoclast precursors. Recent studies (Sömjen et al. 1991; Fenton et al. 1991) suggest that differences in the effects of PTH on bone (stimulation of formation vs resorption) might be due in part to the action of fragments of PTH or PTH-related peptide (PTHrP), since it was shown that certain of these fragments can induce formation while others cause resorption. The insulin-like growth factors (IGF) might also play a role in the induction of bone formation by PTH. IGF-I has been found to stimulate bone formation (Spencer et al. 1991), and Linkhart and Mohan (1989) have shown that PTH stimulates the release of IGF-I and IGF-II from osteoblasts in neonatal mouse calvaria cultures.

PTH secretion is controlled by blood calcium levels (de Winter and Steendijk 1975; Thompson, Baylink and Wergedal 1975; Toffaletti, Cooper and Lobaugh 1991). The mechanism by which PTH produces its effects is only poorly understood, but current thinking is that PTH (as well as other factors) directly stimulates osteoblast-lineage cells, which then exert some influence on osteoclasts, either by production of some soluble "coupling factor" (Rodan and Martin 1981; Shupnik and Tashjian 1982; McSheehy and Chambers 1986; Kahn and Partridge 1987; McSheehy and Chambers 1987; Perry et al. 1987; Thomson, Mundy and Chambers 1987; Fuller, Gallagher, and Chambers 1991; Hoekman et al. 1991; Cheng, Shen, and Peck 1991; Li et al. 1991), or by direct cell/cell contact (Yamashita et al. 1990; Akatsu et al. 1991). Osteoblasts are known to produce a number of factors that affect osteoclast behavior (Heath et al. 1984; Gutierrez, Mundy and Katz 1984; Thomson, Saklatvala and Chambers 1986; Takahashi et al. 1991). Most PTH binding studies have shown that osteoblast-lineage cells are the only bone cells with PTH receptors (Silve et al. 1982; Rouleau, Warshawsky and Goltzman 1986; Rouleau, Mitchell and Goltzman 1990); although several other studies (Rao, Murray and Heersche 1983; Teti, Rizzoli, and Zamboni Zallone 1991; Agarwala and Gay 1992) indicate that osteoclasts might also possess PTH receptors.

PTH has been shown to produce a number of effects in bone cells. PTH stimulates an increase in adenyl cyclase and cyclic AMP (cAMP) activity (Chase, Fedak and Aurbach 1968; Crisp, McGuire-Goldring and Goldring 1984).

Dibutyryl cAMP added to chick tibia cultures increased the effects of PTH (Pandalai and Gay 1990). Peck, Kohler and Barr (1981) found Ca^{2+} to be a positive effector of the cAMP response of isolated bone cells to PTH.

Release of acid hydrolases (Vaes 1968), increase in acid phosphatase activity and hyaluronate synthesis (Luben, Wong and Cohn 1976), and increased RNA synthesis (Bingham, Brazell and Owen 1969) have been shown in osteoclasts under the influence of PTH. In osteoblasts, PTH has been shown to decrease alkaline phosphatase, citrate decarboxylation and prolyl hydroxylase activity. PTH has also been shown to stimulate plasminogen activator production in both osteoclasts and osteoblasts. And finally, Peterson, Heideger and Beach (1985) found that PTH increased the measured electrical potential difference across the periosteal layer in embryonic chick calvaria *in vitro*.

Vitamin D

1,25(OH)₂cholecalciferol [1,25(OH)₂D₃], the most active form of vitamin D, is known to be involved in the control of calcium homeostasis and to influence osteoclast activity. The hormone has been shown to increase calcium absorption by the gut (Garabedian et al. 1974; Nemere, Yoshimoto, and Norman 1984; Kumar 1986) and is implicated in the regulation of renal calcium and phosphate excretion (Reichel, Koeffler, and Norman 1989). 1,25(OH)₂D₃ has also been found to play a role in the control of PTH secretion (Russell, Lettieri, and Sherwood 1986; Reichel, Koeffler, and Norman 1989).

Numerous studies have shown that $1,25(\text{OH})_2\text{D}_3$ causes increases in the number and size of osteoclasts, and in the number of osteoclast nuclei as well as in ruffled border and clear zone area (Holtrop and Raisz 1979; Holtrop et al. 1981; Pharoah and Heersche 1985; Roodman et al. 1985; Grise et al. 1990; Takano-Yamamoto et al. 1992). Hattersley and Chambers (1989) found that D_3 increased the number of CT-receptor-containing cells and the amount of bone resorption at the same time. They concluded that D_3 induces terminal differentiation of osteoclast precursors. Other authors agree that the hormone increases bone or dentine resorption (Holtrop and Raisz 1979; Shiina et al. 1986; McSheehy and Chambers 1987; Takahashi et al. 1988). Grise et al. (1990) were able to show that $1,25(\text{OH})_2\text{D}_3$ increased TRAP and tartrate-resistant acid ATPase (TraATPase) activity in osteopetrotic (*os*) rabbits.

Vitamins D and A have been found to increase collagenase production and to reduce collagenase inhibitor in cultured mouse calvaria, thus changing the collagenase/inhibitor ratio in favor of the active enzyme (Sellers, Meikle, and Reynolds 1980). Heath et al. (1984) later reported that $1,25(\text{OH})_2\text{D}_3$, as well as PTH and PGE_2 , stimulates the secretion of collagenase by osteoblastic cells in mouse calvarial culture. Baylink et al. (1970), and later Underwood and DeLuca (1984), found that vitamin D stimulates bone growth and mineralization indirectly by elevating plasma calcium and phosphorus levels. The $24\text{R},25(\text{OH})_2$ form of the vitamin was found to inhibit ovariectomy-induced bone turnover in dogs (Nakamura et al. 1992).

Receptors for $1,25(\text{OH})_2\text{D}_3$ have been found in osteogenic sarcoma cells (Manolagas, Haussler, and Deftos 1980), in osteoprogenitor cells and osteoblasts (Narbaiz et al. 1983), and in monocytes but not in osteoclasts (Merke et al. 1986).

Estrogen

The decrease in estrogen production at the time of menopause is known to contribute to the bone loss associated with osteoporosis. Estrogen has been shown to play a role in maintaining bone mass. This is at least in part due to an inhibition of osteoclastic bone resorption (Oursler et al. 1991; Hashimoto et al. 1991; Liu and Howard 1991; Oursler et al. 1991b; Chow et al. 1992). This was found to be true in post-menopausal women, where it was shown that the administration of two different estrogen compounds reduced the urinary excretion of calcium and hydroxyproline (indicators of bone resorption) (Selby et al. 1985). These changes were not accompanied by any changes in plasma CT or PTH levels, suggesting that estrogen inhibits bone resorption without the intervention of calcium-regulating hormones. However, Brunner, Schraner, and Wild (1992) reported that progesterone (and testosterone) provoked an increase in nuclear and cell volume and in cell surface, as well as increases in the areas of rough endoplasmic reticulum and the Golgi complex in rat parathyroid glands, indicating that sex steroids might modulate parathyroid secretion.

Several mechanisms for the reduction of bone resorption by estrogen have been proposed. Oursler et al. (1991) found that estrogen increased nuclear

protooncogene mRNA levels in osteoclasts. Liu and Howard (1991) found that estradiol reduced the osteoclast/mm surface length and increased the number of osteoclasts found in the marrow space and separated from bone in female weanling mice. They also reported that estradiol brought about a decrease in osteoclast size, changes in osteoclast morphology, and an increase in the numbers of TRAP+ fragments in the marrow space. Oursler et al. (1991b) showed that 17- β estradiol inhibited bone resorption via its stimulation of TGF- β production.

Estrogen has also been shown to increase bone formation. Hashimoto et al. (1991) showed that estradiol could preserve ectopically-induced bone in mice by enhancing bone formation as well as by inhibiting resorption. Liu and Howard (1991) showed that while estradiol was decreasing osteoclast size and numbers, it also increased the numbers and size of osteoblasts both in trabeculae and along the diaphysis of long bones. Chow et al. (1992) showed that exogenous estrogen could increase bone formation in resorption-inhibited, ovariectomized rats.

Estrogen receptors have been found both in cells of osteoblast lineage (Ohashi, Kusuvara and Ishida 1991) and in osteoclasts (Pensler et al. 1990; Oursler et al. 1991).

Calcitonin (CT)

Calcitonin was so named because it was identified by Harold Copp as a hormone that controlled blood calcium "tone". Much recent evidence has shown that one primary function of calcitonin is to inhibit bone resorption (Friedman, Au and Raisz 1968; Wener, Gorton, and Raisz 1972; Eilon and Raisz 1978;

Wronski et al. 1991). This is thought to happen through an inhibition of osteoclast formation and activity (Kallio, Garant, and Minkin 1972; Lucht 1973; Holtrop, Raisz and Simmons 1974; Feldman, Krieger and Tashjian 1980; Klaushofer et al. 1989; Hunter, Schraer and Gay 1989; Matthews 1992). Eilon and Raisz (1978) showed that calcitonin, as well as cortisol and colchicine, blocked the PTH- and PGE₂-stimulated release of lysosomal enzymes and of ⁴⁵Ca in fetal rat long bone culture. Calcitonin was shown to prevent a decrease in bone volume, and to decrease indices of bone turnover in ovariectomized rats (Wronski et al. 1991). The hormone has also been shown to block the development of ruffled border that is induced by IL-2 and PGE₂. Calcitonin secretion was found to be stimulated by an increase in plasma calcium, and shows a circadian pattern of secretion (Hillyard et al. 1977). Calcitonin has also been found to increase cell proliferation and alkaline phosphatase activity in osteoblast-like cells (Farley et al. 1991).

Calcitonin binding has been demonstrated on osteoclasts (Warshawsky et al. 1980; Rao et al. 1981; Nicholson et al. 1986; Eliam et al. 1988) and on osteoclast precursors (Nicholson et al. 1986; Taylor et al. 1989; Minkin and Yu 1991). There are also CT binding sites in the kidney (Warshawsky et al. 1980). Rao et al. (1981) reported sCT binding to periosteal osteoblasts, as well as to striated muscle fibers and erythrocytes.

When calcitonin is administered over a period of days, the hormone suddenly seems to lose its ability to inhibit osteoclastic activity. This phenomenon is

termed "escape". Wener, Gorton and Raisz (1972) reported that CT inhibited PTH- or 25(OH)D₃-induced bone resorption *in vitro*, but after several days of treatment CT lost its inhibitory effect. They found that escape could be prevented or delayed by decreasing the concentration of calcium or phosphorus in the culture medium. Feldman, Krieger, and Tashjian (1980) suggested that escape was due to the fact that calcitonin inhibits the fusion of preexisting mononuclear osteoclast precursors with existing osteoclasts, thus making osteoclast recruitment dependent on the fusion of newly formed postmitotic precursor cells - a slow process which could account for the lag time in osteoclast activity followed by "escape". Klaushofer et al. (1989) noted the escape phenomenon in a cultured neonatal mouse calvaria system. They found that escape occurred coincidentally with the appearance of small active osteoclasts, and suggested that escape occurred when a new batch of osteoclasts differentiated to take the place of the already-existing osteoclasts that had been inhibited. This would agree with the theory proposed by Feldman, Krieger, and Tashjian (1980).

Other hormones

Other hormones such as growth hormone (Takagi et al. 1992) and tri-iodothyronine (Allain et al. 1992) have been shown to affect osteoclastic activity, but their roles in osteoclast function have not yet been thoroughly examined.

Paracrine Regulators of Osteoclast Function

Colony Stimulating Factors

The development and differentiation of hemopoietic stem cells into specific

cell lineages has been intensively studied. It has been recognized that "hemopoietic cells are intrinsically incapable of unstimulated cell division" (Metcalf 1985), but certain glycoproteins are capable of stimulating development of hemopoietic cell colonies *in vitro*. These substances have been called **colony stimulating factors**. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) appear to be involved in osteoclast development and activity. However, there is debate over the role of these factors: some authors report stimulation of osteoclast development with these factors, while others report inhibition.

Shinar, Sato, and Rodan (1990) found that generation of osteoclasts in culture was inhibited by GM-CSF and M-CSF (as well as by IL-3), but not by granulocyte-CSF (G-CSF), IL-6 or leukemia inhibitory factor (LIF). The authors suggested that the three factors causing inhibition of osteoclast formation might either be diverting a common precursor cell to some other developmental pathway and thus reducing its availability for the formation of osteoclasts, or that the cells generated in response to these factors, especially macrophages, might release cytokines which inhibit the formation of osteoclasts. Hattersley et al. (1991b) reported that M-CSF in normal (as opposed to osteopetrotic) mouse spleen cell cultures stimulated macrophage production but inhibited osteoclast formation. However, in spleen cell cultures from osteopetrotic *op/op* mice, a mutant strain that lack osteoclasts, M-CSF restored osteoclast production.

Takahashi et al. (1991) cocultured spleen cells from *op/op* mice with osteoblast cells from calvaria of normal mice. They found that TRAP+ multinucleated cells (MNC) formed in the presence of D_3 . But when osteoblasts from *op/op* mice were cocultured with spleen cells of normal mice, no TRAP+ MNCs formed unless M-CSF was added. These results suggest that the defect in *op/op* mice is the lack of production of M-CSF by osteoblasts, and that M-CSF from osteoblasts is needed to produce osteoclasts. In a similar experiment, Kodama et al. (1991) established a clonal stromal cell line that supports hemopoiesis with neonatal *op/op* mouse calvaria. Adding M-CSF to the coculture increased the number of cells identified as macrophages by the authors. TRAP+ cells appeared when both M-CSF and D_3 were added and when stromal cells were in contact with the *op/op* cells.

Marks and colleagues used a different osteopetrotic mutation, the toothless (*tl*) rat, to demonstrate the effects of CSF. In one study (Marks et al. 1992) it was found that injecting CSF-1 into rats during the period from birth to 6 weeks of age reduced osteopetrosis, increased body weight and brought about tooth eruption and the induction of large numbers of osteoclasts and macrophages. In another study (Iizuka et al. 1992), the success of CSF-1 in stimulating tooth eruption in *tl* rats was found to be inversely related to the age at which treatment began. Staining by TRAP and TraATPase was found to be reduced or negligible in untreated *tl* rats, heavy in normal rats and of intermediate intensity in

CSF-1 treated *tl* rats. Treated rats were also shown to have a greater number of osteoclasts on alveolar bone than untreated animals.

Corboz et al. (1992) reported that human recombinant M-CSF stimulates bone resorption *in vitro* only when the resorption is dependent on the generation of new osteoclasts. M-CSF does not appear to stimulate mature osteoclasts.

Cytokines

The cytokines are soluble products produced by certain cells (lymphocytes, osteoblasts, etc.) that influence growth, morphology and other specific functions of the same or other cells in their local environment (Krane, Goldring and Goldring 1988). Many of these products have been shown to affect osteoclast development and activity.

Interleukins (IL)

One of the most important of the cytokines in regulating osteoclast activity is IL-1, a substance produced by monocytes and macrophages.

Most studies agree that IL-1 increases bone resorption (Gowen and Mundy 1986; Thomson et al. 1986; Akatsu et al. 1991; Nguyen et al. 1991). Pfeilschifter et al. (1989) and Akatsu et al. (1991) reported increases in the number of osteoclasts or MNCs with IL-1. Several studies show that osteoblasts are necessary to stimulate IL-1-induced osteoclast formation and bone resorption (Thomson et al. 1986; Akatsu et al. 1991), but Nguyen et al. (1991) reported that IL-1 caused a decline in serum osteocalcin levels and in the mineral apposition

rate (which are measures of osteoblastic activity). Gowen and Mundy (1986) found that cyclooxygenase inhibitor did not inhibit IL-1-stimulated bone resorption, implying that prostaglandins do not mediate IL-1 activity, but Akatsu et al. (1991) found that the increase in osteoclast numbers and in bone resorption could be inhibited by indomethacin, indicating that prostaglandin synthesis is involved in IL-1 activity. Similarly, Linkhart and MacCharles (1992) reported that indomethacin inhibits the IL-1-stimulated release of IGF-I and Ca from neonatal mouse calvaria in culture, showing that IL-1 acts via prostaglandin synthesis. Several studies (Ikeda et al. 1988; Evans, Bunning, and Russell 1990) reported that IL-1 β caused an increase in PGE₂ production by osteoblast-like cells.

IL-4 has been reported by Shioi et al. (1991) to inhibit the formation of osteoclasts in cocultures of bone marrow cells and stromal cells. This inhibitory effect of IL-4 was shown to be antagonized by an IL-4 monoclonal antibody.

IL-6 is another interleukin that has been shown to stimulate MNC formation in marrow culture (Roodman et al. 1992). It was found both in the marrow plasma and blood serum of patients with Paget's disease.

Transforming growth factors (TGF)

Two types of transforming growth factors, *alpha* and *beta*, have been identified as affecting bone cell activities. TGF-*alpha* has been found to induce bone resorption (Ibbotson et al. 1985) by increasing the proliferation of osteoclast precursors (Takahashi et al. 1986) and by stimulating PGE₂ production (Tashjian

et al. 1985). It has also been shown that TGF-*alpha* has additive effects with PTH or PTHrP in stimulating bone resorption and adenylate cyclase activity in osteoblast-like cell line cultures (Rosol et al. 1991).

The role of TGF- β seems more complex. Like TGF-*alpha*, TGF- β has been shown to increase bone resorption (and PGE₂ production) in some circumstances (Tashjian et al. 1985). Mundy and Bonewald (1990) attribute this increase in bone resorption to the stimulation of prostaglandin synthesis by TGF- β , with the prostaglandin then being the stimulating agent for bone resorption. Some studies have also shown TGF- β to be inhibitory to bone growth or alkaline phosphatase activity in culture (Noda and Rodan 1986; Rosen et al. 1988). However, other studies have found that TGF- β increases alkaline phosphatase activity (Noda and Rodan 1987) and the synthesis of mRNAs which code for various osteoblast products such as procollagen and osteonectin (Noda and Rodan 1987; Penttinen, Kobayashi and Bornstein 1988; Strong et al. 1991), as well as DNA synthesis (Hill et al. 1986; Centrella, McCarthy and Canalis 1988) and collagen production and ³[H]-thymidine incorporation (Hill et al. 1986; Hock, Centrella, and Canalis 1988; Centrella, McCarthy and Canalis 1987). Evidence also suggests that TGF- β increases the rate of synthesis of ascorbate transporter proteins or regulatory proteins. Dixon and Wilson (1992) found that ascorbate transport was increased in UMR-106 (osteoblast-like) cell cultures which had been pretreated with TGF- β , but that this could be blocked by cycloheximide, a protein synthesis inhibitor. Several studies showed a biphasic effect of TGF- β depending on its dosage

(Centrella, McCarthy and Canalis 1988) or the age of the fetuses producing the TGF- β (Hill et al. 1986).

Robey et al. (1987) showed that TGF- β might act as an autocrine factor in fetal bovine bone-forming cells. These cells were found to express the TGF- β receptor and to respond to TGF- β with increased growth. TGF- β was found in the conditioned medium from these cells which the authors identified as osteoblasts.

Tissue necrosis factor (TNF) is another cytokine that affects osteoclasts. Van der Pluijm et al. (1991) found that TNF- α did not alter activity of mature osteoclasts as measured by ^{45}Ca release from fetal mouse long bone, but that in fetal mouse bone it stimulated resorption at low concentrations and inhibited resorption at high concentrations.

Other growth factors

Epidermal growth factor (EGF) has been shown to affect bone resorption. Raisz et al. (1980) found EGF to increase release of ^{45}Ca from cultured fetal rat long bone shafts. Indomethacin did not block this phenomenon. However, Shupnik and Tashjian (1982) found that EGF increases PGE_2 production by osteosarcoma cells. Takahashi et al. (1986) showed that EGF can stimulate bone resorption by increasing the proliferation of osteoclast precursors.

Tashjian et al. (1982) showed that platelet-derived growth factor (PDGF) caused an increase in PGE_2 production and bone resorption as evidenced by an

increase in culture-medium calcium concentration. They suggested that PDGF stimulates bone resorption via enhanced PGE_2 production.

Spencer et al. (1991) found that when insulin-like growth factor (IGF-I) was continuously infused into the right hindlimb arterial supply of rats, using the other leg as a control, IGF-I induced bone formation, stimulated osteoblastic activity, and decreased the number of osteoclasts. However, Sloodweg et al. (1992) recently reported that when IGF-I was added to mouse bone cultures containing osteoclast precursors, the number of osteoclasts increased, as did bone resorption. Thus, while IGF-I seems to be primarily anabolic in its effects, it appears to induce bone resorption under certain circumstances.

Scheven et al. (1991) isolated what they called osteoclast growth factor (OGF) from fetal rat calvaria culture medium. The conditioned medium (containing OGF) increased the number of osteoclasts in fetal long bone cultures, but did not exert a direct effect on bone resorption by pre-existing osteoclasts. In bone marrow cultures conditioned medium stimulated formation of TRAP+ mononuclear cells. Peritoneal macrophages did not respond to conditioned medium. Antibody to GM-CSF did not prevent conditioned medium activity.

Prostaglandins (PG)

The prostaglandins are a ubiquitous group of compounds derived from arachidonic acid. Their activities are varied and complex, but they have been shown to play a definite role in the activation or inhibition of bone cells.

Much research has shown that PGs mediate bone resorption. Franklin and Tashjian (1975) found that continuous i.v. infusion of PGE_2 in rats produced elevated plasma calcium and phosphate levels, suggesting a breakdown of bone. Several studies (Rifkin et al. 1980; Schelling et al. 1980) demonstrated that PGE in bone cultures caused an increase in calcium release and in osteoclast number as well as in ruffled border development. Indirect evidence, using indomethacin to inhibit PG synthesis, suggests that prostaglandins mediate the bone resorption induced by collagenase (Dowsett et al. 1976), by complement (Sandberg et al. 1977), by EGF (Tashjian and Levine 1978), by monocytes or monocyte-conditioned medium (Dominguez and Mundy 1980), by PDGF (Tashjian et al. 1982) and by IL-1 (Garrett and Mundy 1989; Linkhart and MacCharles 1992). Lerner (1980) found that indomethacin decreased the release of ^{45}Ca , Ca^{2+} , P_i , β -glucuronidase, β -galactosidase, and β -N-acetylglucosaminidase, as well as glucose consumption and lactate production in neonatal mouse calvarial organ culture. In addition, one study (Minkin et al. 1981) found that tumor extracts from patients with humoral hypercalcemia of malignancy (HHM) caused ^{45}Ca release in neonatal mouse calvaria cultures, and that this could be inhibited by indomethacin and eicosatetraynoic acid, indicating the involvement of prostaglandins. Several studies have shown that cAMP might mediate the effects of prostaglandins (Tashjian et al. 1982; Akatsu et al. 1989).

PGE_1 given subcutaneously was shown by Yonaga and Morimoto (1979) to inhibit longitudinal bone growth of the proximal tibia and longitudinal and

appositional growth of the incisal dentin. They reported that the actions of PGE_1 were identical to those of CT and they felt that calcitonin might mediate the effects of PGE_1 . PGI_2 as well as dibutyryl cAMP were shown to produce identical effects to those of calcitonin in inhibiting bone resorption and osteoclast motility. Theophylline, which inhibits intracellular cAMP degradation, potentiated these effects, suggesting that CT and PGI_2 act by increasing cAMP activity.

Although most studies have shown that prostaglandins stimulate bone resorption, the resorption of bovine bone slices by isolated osteoclasts in culture was found to be inhibited by several prostaglandins in a study by Fuller and Chambers (1989).

Several studies have provided evidence that prostaglandins can also stimulate bone formation. Marks and Miller (1988) showed that infusion of PGE_1 into alveolar bone in dogs over a period of five weeks caused increased bone formation when compared to controls. Li et al. (1990) injected dogs subcutaneously once a day with PGE_2 for 31 days and found a net increase in cancellous and cortical bone formation in iliac crest and mid-tibial shaft. And Flanagan and Chambers (1992) reported that PGE_1 and PGE_2 increased bone nodule formation in rat calvarial cell cultures.

Miscellaneous Compounds

Various other endogenous substances have been found to affect osteoclast morphology and activity, confirming the idea that the control of the osteoclast is

very complex. Some of these substances and their actions will be briefly discussed below.

Matsui et al. (1991) found that in sheep fed a protein-deficient diet the number of osteoclasts and the TRAP activity were lower than in control animals. Administration of D_3 could not rectify the problem.

Plasminogen activator, produced by osteoblasts, is a factor implicated in the induction of bone resorption. Hamilton et al. (1984) found that PTH, PGE_2 , EGF and $1,25(OH)_2D_3$ stimulated PA activity in osteoblasts. Hoekman et al. (1991) reported that bPTH(1-84), hPTH-like protein(1-34), PGE_2 , IL-1 beta, and TNF-alpha stimulated production of plasminogen activators by three different osteoblastic cell lines, whereas TGF-beta reduced PA production. Cheng et al. (1991) examined the effects of various growth factors and cytokines on secretion of PA and plasminogen activator inhibitor (PAI) in osteoblast-enriched calvarial cell cultures. Fibroblast growth factor (FGF), EGF and PDGF increased PA in culture medium and cell layer extract. IL-1 alpha, TNF-alpha and IGF-1 caused no change in PA activity. TGF-beta increased PA activity in some assays, and also increased PAI activity. Basic FGF and PDGF slightly increased PAI secretion. The authors concluded that the regulation of PA activity by growth factors and cytokines did not correlate with their resorbing/stimulating activities. Thus PA secreted by osteoblasts appears to be only one of several factors involved in initiation of bone resorption.

Nitric oxide has recently been implicated in osteoclast inhibition. MacIntyre et al. (1991) using an isolated osteoclast culture system, found that nitric oxide produced a decrease in osteoclast spread area by about 50%.

Miyauchi et al. (1991) found that osteopontin binding to osteoclast $\alpha_v\beta_3$ integrin causes a calmodulin-dependent reduction in cytosolic Ca^{++} , which is thought to participate in the regulation of osteoclast function.

Mundy and Poser (1983) found that osteocalcin (BGP) has chemotactic activity for breast cancer cells, monocytes and rat osteosarcoma cells. They suggested that since BGP is released during bone turnover, it might be a mechanism of recruiting cells for bone remodelling. Glowacki et al. (1991) implanted apatite, either alone or containing osteocalcin, BSA or collagen, subcutaneously into rats. Only the particles containing osteocalcin stimulated production of MNCs that were TRAP+ and that had osteoclast morphology (ruffled border, clear zone).

Ljunggren et al. (1991) examined the role of polyamines (putrescine, spermidine, spermine) in PTH-induced bone resorption. They found that PTH stimulated ornithine decarboxylase activity in cultured osteoblast-like cells, and caused a modest increase in putrescine and spermidine. However, polyamine synthesis inhibitors had no effect on ^{45}Ca release, but polyamines could inhibit ^{45}Ca release directly.

Malone et al. (1982) showed that type I collagen, $\alpha_2\text{HS}$ glycoprotein and osteocalcin evoke a chemotactic response in human monocytes (which they supposed to be osteoclast precursors).

Oreffo et al. (1988) showed that retinol or retinoic acid increased cellular adhesion to substratum, acid phosphatase activity and release of $^3\text{[H]}$ proline as evidence of increased resorption in isolated chicken osteoclast cultures.

Fatemi et al. (1991) showed that magnesium deficiency in human subjects caused a fall in serum calcium and serum D_3 . PTH either fell or did not change, showing an impaired PTH secretion.

Pharmacological Compounds

The bisphosphonates are a class of pharmacological compounds that have been shown effective in reducing bone resorption. Miller et al. (1977) found that a series of ethane-1-hydroxy-1,1-diphosphonate (EHDP) injections resulted in increases in the number of osteoclasts, osteoclast nuclei and osteoclast nuclei per profile, and in the rate of osteoclast production from precursors, but at the same time slowed bone resorption.

Carano et al. (1990) showed that several bisphosphonates reduced bone resorption in cultured chicken osteoclasts, and reduced H^+ accumulation in osteoclasts as measured by acridine orange staining. They also reduced osteoclast protein synthesis. The authors suggested that bisphosphonates reduce resorption by metabolic inhibition of the target osteoclasts, because the bisphosphonates bind to bone mineral and prevent osteoclast attachment and resorption.

Sato et al. (1991) injected $^3\text{[H]}$ alendronate, another bisphosphonate, into newborn rats. Osteoclast surfaces and bone surfaces were labelled. Osteoclasts from adults animals infused with PTHrP and injected with alendronate lacked

ruffled border but not clear zone. Alendronate bound to bone particles *in vitro* and inhibited bone resorption by isolated chicken or rat osteoclasts when the bone surface had alendronate at certain concentrations bound to it.

Bonucci et al. (1992) showed that ipriflavone has a direct inhibitory effect on bone resorption. They suggest that this is due to reduction in osteoclast recruitment or differentiation as evidenced by histomorphometric techniques.

Osteoblast Involvement in Osteoclast Activation

Many studies are pointing to an active role by osteoblasts in the recruitment and activation of osteoclasts. Several recent findings, in addition to studies already cited, are given here.

Udagawa et al. (1989) showed that when osteoblast-like cell lines were cultured with mouse spleen cells, osteoclast-like TRAP+ cells appeared and resorbed whale dentine. Yamashita et al. (1990) isolated several cell lines from fetal mouse calvaria and cocultured them with murine spleen cells. PTH increased cAMP in these osteoblast-like cells, and PTH + IL-1 also induced TRAP+ MNC formation in this coculture system. Contact between these (KS-4) cells and spleen cells was necessary for osteoclast formation to take place.

Greenfield et al. (1992) reported that conditioned medium from avian osteoblast cultures could stimulate bone resorption and the expression of the specific osteoclast antigen 121F, which they attributed to interaction of an osteoblast-produced factor with osteoclast precursors. Hiura et al. (1991) found that when spleen cell cultures were treated with conditioned medium from a

clonal osteoblast cell line taken from "young" cells, MNC formation was inhibited in the presence of D_3 and GM-CSF, but when the conditioned medium was taken from older cells, MNC formation was stimulated. PGE_2 was detected in the conditioned medium from both "young" and "old" cells, and the inhibition induced by the younger cells was partially abolished by indomethacin. 60-day conditioned medium and D_3 stimulated MNC development even in the absence of GM-CSF, but was inhibited by antibody to GM-CSF. The authors concluded that osteoblasts produce GM-CSF which stimulates MNC formation, and PGE_2 which inhibits MNC formation, and they suggested that different stages of osteoblast development determine the effects of the conditioned medium on osteoclast development.

Fuller et al. (1991) proposed that osteoblasts mediate osteoclast activation by releasing "osteoclast resorption-stimulating activity" (ORSA), and that this product is bound either to glycosaminoglycans (GAG) of bone matrix or to the osteoblast cell surface. They suggested that this immobilization of ORSA by GAG might aid the osteoblasts in regulation of osteoclast activity.

The Calcium Deficiency Model

The calcium deficiency model has been an effective tool in studying bone cell dynamics *in vivo*. Giving rats a calcium-deficient diet or replenishing calcium stores lost due to deficiency causes a shift in the bone resorption/bone formation equilibrium as the populations and activities of osteoclasts and osteoblasts are

modulated. This model also gives insight into the control of bone metabolism by local and systemic humoral factors.

Some early studies (Stauffer et al. 1972; Stauffer et al. 1973) showed that feeding rats a calcium-free diet for up to twelve days resulted in an increase in the medullary area of the tibia in cross-section. This was accompanied by hypocalcemia, inhibition of bone formation, increased bone resorption and an increase of parathyroid gland volume. These findings agreed with those of de Winter and Steendijk (1975) who reported a decrease in cortical bone area in the mid-shaft of the femur during calcium-deficiency in lactating rats, with bone losses in the endosteum and in the spongiosa. It was also reported that mineralization during this deficiency period was delayed, and the rate of mineral deposition was decreased (Stauffer et al. 1973). Weinreb, Rodan and Thompson (1991) showed an elevation in serum PTH with calcium deficiency, although serum calcium was not affected. They also reported a reduction in femoral ash weight, in tibial cortical thickness and in trabecular bone volume in calcium-deficient animals.

Giving calcium-deficient animals a normal calcium-containing diet caused a decrease in medullary area as bone formation replaced bone resorption, but it took months to replace the amount of bone that was lost in a 1- to 2-week calcium deficiency period (Stauffer et al. 1972; Drivdahl, Liu and Baylink 1984). Stauffer et al. (1972) also reported that the increased bone formation during calcium repletion took place primarily in areas where resorption had taken place

during calcium deficiency. De Winter and Steendijk (1975) also found that, while calcium repletion increased the amount of bone formation, the cortical bone area did not return to normal after 3 weeks calcium repletion. They discovered that bone density in the newly-formed bone was lower than in normal animals.

The increase in resorption during calcium deficiency can be attributed to an increase in osteoclast numbers, size, and number of nuclei (Thompson, Baylink and Wergedal 1975). However, the number of osteoblasts has also been reported to increase in proportion to the number of osteoclast nuclei, thus indicating a coupling mechanism between resorption and formation. Liu, Baylink and Wergedal (1975) found that neither calcitonin, PTH, nor mechanical stress was necessary to bring about repletion in calcium-deficient rats, but that the rate of bone formation during repletion was related to the seriousness of the calcium depletion during the deficiency period.

Hughes et al. (1975) demonstrated that low calcium or low phosphorus diets increased the concentration of circulating $1,25(\text{OH})_2\text{D}_3$, but while the effect of calcium deficiency on the D_3 response was dependent on intact parathyroid glands, the response due to phosphorus deficiency was independent of these glands.

Liu, Baylink and Wergedal (1978) revealed that calcium repletion is not dependent on mechanical stress. They rendered one hind limb immobile in a group of rats by transecting the sciatic nerve, and found that there was no difference in the rate of repletion between immobilized animals and normal

animals. However, Weinreb, Rodan and Thompson (1991) found that immobilization of a limb due to nerve transection did cause additional loss of bone mass, and an increase in osteoclast numbers beyond what could be accounted for by calcium deficiency alone.

Liu, Ivey and Baylink (1981) examined the effects of vitamin D deficiency on calcium repletion. They found that although D deficiency caused serious hypocalcemia and elevated serum PTH, these factors did not prevent repletion. In fact, there was a depression in the resorption rate of repleting animals showing that PTH-mediated bone resorption was being suppressed. Vitamin D-deficient animals did show some impairment in mineralization.

McMillan et al. (1989) demonstrated that giving a calcium-containing diet to deficient rats caused rapid changes in osteoclast morphology suggesting increased osteoclast inhibition. They found that within three hours of the beginning of a calcium-containing meal, decreases in the ruffled border index and the osteoclast contact index could be measured. This was accompanied by increases in the osteoclast isolation index, showing that other cells were already beginning to insert themselves between the osteoclasts and the bone surface. (For definitions of these indices, see p. 38)

Sissons, Kelman and Marotti (1985) made use of the calcium deficiency model to show that there was no increase in the size of osteocyte lacunae with calcium deficiency, suggesting that osteocytic osteolysis did not take place.

Effects of Fasting

Many of the studies concerning fasting have dealt with weight loss in obese people, or have focused on changes in carbohydrate, lipid and protein metabolism (eg., Young and Scrimshaw 1971). While experiments examined such topics as the effects of fasting on liver and kidney metabolism (Owen et al. 1969), pancreas (Webster et al. 1972), and magnesium depletion (Drenick, Hunt and Swendseid 1969), little is known about the effects of fasting on bone cell activity.

One study dealing with calcium homeostasis (Wong and Klein 1984) showed that there is a circadian shift between bone and diet (i.e., intestine) as sources of blood calcium in dogs, with bone normally accounting for two thirds of the blood calcium and diet providing one third. Dogs were given multiple injections of ^{45}Ca over a period of six weeks, with experiments starting four weeks after the end of labeling. In dogs on normal calcium-containing diets, the concentration of ^{45}Ca in serum fluctuated following a circadian pattern, but the total serum calcium level remained constant. The authors found that when dogs were put on a low calcium diet or fasted, the labelling of blood calcium was high, and the level of serum ^{45}Ca no longer fluctuated, indicating that bone was providing essentially all of the blood calcium. In thyroparathyroidectomized dogs, however, the labelling of blood calcium was low and did not fluctuate, showing that calcium derived from intestinal absorption had become the primary source of serum calcium.

Focus of This Study

Data reported by Liu et al. (1982) showed that virtually all osteoclasts that had been recruited to tibial endosteum by 12 days of dietary calcium deficiency disappeared following 24-72 hours of dietary calcium supplementation. Together with the finding by McMillan et al. (1989) that a change in osteoclast morphology suggesting a reduction in osteoclast activation is apparent after as little as three hours following calcium supplementation, it was concluded that at some time point between 3 hours and 24 hours, osteoclasts were being "shut down" and were withdrawing from the endosteum. Consequently, the hypothesis was formulated that there exists some temporal and/or quantitative dietary calcium threshold beyond which osteoclasts will be completely inhibited and will recede from bone, and that once osteoclasts have reached this point, a certain minimum time will be required for osteoclast re-recruitment to bone.

A secondary hypothesis was generated as a result of the first experiment in this study. The data from this experiment suggested that fasting had reversed the morphological changes brought about by calcium supplementation; therefore, the hypothesis was proposed that fasting can reactivate inhibited osteoclasts. It was further proposed that fasting might be effective in initially recruiting osteoclasts.

The goal of this study, then, was to examine the modulations of osteoclast morphology following changes in dietary calcium supplementation in calcium-deficient rats. This study has been divided into two parts; the first part examines the dietary control of osteoclast recession (withdrawal from bone), and the second

part focuses on how dietary factors (fasting and calcium deficiency) influence the osteoclast recruitment or activation processes.

MATERIALS AND METHODS

In all experiments, male Sprague-Dawley rats, aged 21-28 days, beginning weight approximately 50 g, were used. Animals were housed either individually or in groups. The animals were put on a 12/12 light/dark cycle; in some of the studies a reversed light cycle was used to facilitate the execution of the feeding protocol. Animals that were put on a reversed lighting cycle (lights on nights, lights off days) were fed standard lab chow during a six-day period of habituation to the new lighting cycle. Otherwise all animals were fed either a custom normal calcium diet containing 0.7% phosphorus and 0.7% calcium, or a custom calcium free diet with 0.4% phosphorus and 0.0% calcium (ICN Biochemicals, Cleveland) (see Appendix A for diet composition), and tap water *ad libitum*. At the completion of each experiment, the animals were deeply anesthetized either by inhaling ether or by i.p. injection with 50% urethane, then killed by perfusion with a glutaraldehyde fixative (1.75% glutaraldehyde, 0.08 M sucrose, 0.05 M cacodylate buffer, pH 7.3). Tibia were removed, stripped of soft tissues, and decalcified in a solution of 3.7% EDTA, 5% glutaraldehyde, 0.033 M cacodylate buffer, pH 7.3, and 6.85% sucrose for one to two weeks. The tibias were then cut into 2 mm segments and washed three times at 15 minutes each in 0.1 M cacodylate buffer, pH 7.3, followed by 2 hours in 1% OsO₄ in cacodylate buffer. The bone fragments were next dehydrated in a graded acetone series, changed to propylene oxide (2 treatments at 15 min. each), and put in 1:1 propylene oxide:epon (Polybed 812) for 3 hours. The tissues were changed to a 1:2 propylene oxide:epon mixture and left overnight, then embedded in epon. One

μm tibial cross-sections were cut using a Porter Blum Ultramicrotome MT-2. The sections were stained for 5 minutes with a warm solution of 5 parts 1% toluidine blue/1% azure II in 4 parts 5% Na_2CO_3 , rinsed three times in H_2O , then mounted on slides and heat-dried. Cover slips were mounted with Permount.

Morphometric Analysis

A total of three tissue sections, one section from each of three blocks, were examined per animal using the DATAVOICE computerized voice command data collection system. All slides were examined at 1000 x with a Zeiss light microscope equipped with a *camera lucida*, and using a digitizing pad to register x,y coordinates. This program made it possible to simultaneously measure the total length of the endosteal surface in these tibial cross-sections, and determine the various bone morphological indices which are described below. These indices are based on the stereological principle that the length of the boundary of a feature in a two-dimensional tissue section is proportional to the surface area of that feature in three dimensions.

Morphological Indices:

Osteoblast Area Fraction (OBAF): The percentage of the total length of endosteal surface covered by osteoblasts.

Resting Area Fraction (RAF): The percentage of the total length of endosteal surface covered by resting/bone lining cells.

Osteoclast Area Fraction (OCAF): The percentage of the total length of endosteal surface covered by osteoclasts. The OCAF can be subdivided into the following categories:

Ruffled Border Index (RBI): The percentage of the osteoclast/bone interface demonstrating ruffled border.

Contact Index (CI): The percentage of the osteoclast/bone interface demonstrating physical bone/cell contact (including ruffled border).

Separation Index (SI): The percentage of the osteoclast/bone interface demonstrating a gap (as determined visually) between part of the cell and bone.

Isolation Index (II): The percentage of the osteoclast/bone interface where the osteoclast is partially isolated from the bone by another cell type.

Removed Index (REM): The percentage of the total endosteum demonstrating osteoclasts completely removed from the bone surface and separated from the bone by other cell types. (This index is calculated separately from all other measurements.)

Statistical Analysis

The various treatment groups in each experiment were compared by ANOVA using the CRISP statistical package (Crunch Software Corporation, San Francisco). The p values for the ANOVA are based on the Fmax ratio, and post-hoc t-tests were used for comparisons between groups within each experiment.

RESULTS

Dietary Control of Osteoclast Recession

The first three experiments in this study examined the induction of osteoclast recession from bone by calcium supplementation, and examined the effects of fasting on this osteoclast recession.

Experiment #1: **Inhibition of Osteoclasts by Dietary Calcium in Calcium-Deficient Rats is Graded**

It was shown by Liu et al. (1982) in calcium-deficient rats, that receiving calcium-containing diet for 1 to 3 days caused virtually all osteoclasts to disappear from the endosteal surface of the tibial diaphysis. McMillan et al. (1989) showed that this inhibition first becomes morphologically apparent after as little as three hours of calcium supplementation. Apparently, at some point between 3 hours and 24 hours, osteoclasts were being totally inhibited. Two alternatives were considered: first, that osteoclast inhibition might require a certain dietary calcium threshold, and that when this threshold was reached, one would observe a rapid decline in the number and activity of osteoclasts until all osteoclasts had virtually disappeared. The second option was that osteoclast inhibition was graded, that is, that with increasing dietary calcium supplementation, morphological indices would gradually change in a dose- and/or time-dependent manner, demonstrating increased osteoclast inhibition, until the osteoclasts had completely withdrawn from bone. The goal of the first experiment was to test the hypothesis that osteoclast inhibition was a graded phenomenon rather than a "threshold" phenomenon.

Seven groups of 6 rats were used. Animals were kept in a darkened room and subjected to a reversed light cycle (lights on 21:00, light off 9:00). The animals were given six days to adjust to the reversed lighting before beginning the experiment. During this time the rats were given lab chow *ad libitum*. After this habituation period, animals were fed either the custom normal calcium diet or the custom calcium free diet *ad libitum* during the dark period of the light/dark cycle. On day eight, the animals were given 6 grams of diet divided into five 1.2 g feedings three hours apart. Each group followed an individual feeding protocol:

Normal controls: received only normal calcium diet for eight days

Deficient controls: received only calcium free diet for eight days

3-hour supplemented: received calcium free diet seven days, normal calcium diet on day 8 for first feeding only, with calcium free diet for remaining feedings

6-hour supplemented: received calcium free diet seven days, normal calcium diet on day 8 for first two feedings only, with calcium free diet for remaining feedings

9-hour supplemented: received calcium free diet seven days, normal calcium diet on day 8 for first three feedings only, with calcium free diet for remaining feedings

12-hour supplemented: received calcium free diet seven days, normal calcium diet on day 8 for first four feedings only, with calcium free diet for the remaining feeding

Total supplemented: received calcium free diet seven days, normal calcium diet on day 8, all feedings

The animals were killed at 9:00 on the day following the last feeding (day 9) and tissues prepared as described above for morphological analysis.

In this and all subsequent experiments, there were no significant differences in amount of food consumed or weight gained between experimental groups. Table 1 (page 43) shows the means \pm SEM for the 8 parameters measured. A comparison of the normal and the deficient control groups using the t-test showed significant differences between the two groups in 4 of the 8 morphological indices: OBAF (normal > deficient; $p=0.003$), OCAF (deficient > normal; $p=0.002$) (see Figure 1, page 44), CI (normal [mean = 93.1; SD = 3.5] > deficient [mean = 87.9; SD = 3.8]; $p=0.048$), and II (deficient > normal; $p=0.032$) (see Figure 2, page 45). When the deficient control group was compared with the experimental groups using ANOVA, no significant differences could be found between groups, although there was evidence of a trend toward the reduction of the ruffled border index with increasing amounts of normal calcium diet (see Figure 3, page 46).

In this experiment, it had been expected that increasing amounts of calcium supplementation would produce changes in osteoclast morphology indicating an increased tendency toward recession from bone. This increased recession would be manifested by a decrease in the ruffled border index, and by a decrease in the percentage of the osteoclast/bone interface characterized by actual contact of the osteoclast with the bone surface (CI), as well as by a decrease in the percentage of the endosteal surface covered with osteoclasts (OCAF). This should have been accompanied by an increase in the II and in the SI. It was also hypothesized that there would be an increase in the percentage of osteoclast profiles completely removed from the bone (REM).

GROUP	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
N.C.	36.6 \pm 5.7	53.2 \pm 5.1	10.2 \pm 4.8	15.4 \pm 5.5	93.1 \pm 3.5	2.0 \pm 3.2	4.9 \pm 2.3	0.1 \pm 0.2
D.C.	19.0 \pm 8.0	48.8 \pm 6.3	32.2 \pm 9.5	19.2 \pm 4.1	87.9 \pm 3.8	4.2 \pm 3.3	7.9 \pm 1.0	0.6 \pm 0.5
3 Hr	25.1 \pm 11.7	53.1 \pm 9.3	21.8 \pm 6.9	22.4 \pm 6.7	88.1 \pm 3.3	4.5 \pm 2.0	7.4 \pm 2.9	0.1 \pm 0.2
6 Hr	27.2 \pm 12.9	42.5 \pm 9.3	30.4 \pm 9.2	19.0 \pm 5.2	89.9 \pm 4.9	3.2 \pm 2.7	6.9 \pm 2.3	0.3 \pm 0.5
9 Hr	22.3 \pm 5.9	52.7 \pm 4.1	25.1 \pm 7.1	11.9 \pm 6.0	86.7 \pm 6.1	6.9 \pm 4.9	6.4 \pm 1.6	0.6 \pm 0.7
12 Hr	22.1 \pm 11.6	56.6 \pm 12.5	21.3 \pm 4.7	16.3 \pm 6.1	92.2 \pm 1.4	3.2 \pm 1.6	4.6 \pm 2.2	0.3 \pm 0.4
TOT	23.1 \pm 11.5	51.8 \pm 10.7	25.1 \pm 3.5	11.5 \pm 6.2	89.9 \pm 5.7	2.3 \pm 1.1	7.7 \pm 4.8	0.1 \pm 0.1

TABLE 1: Data for Experiment #1, means \pm SD

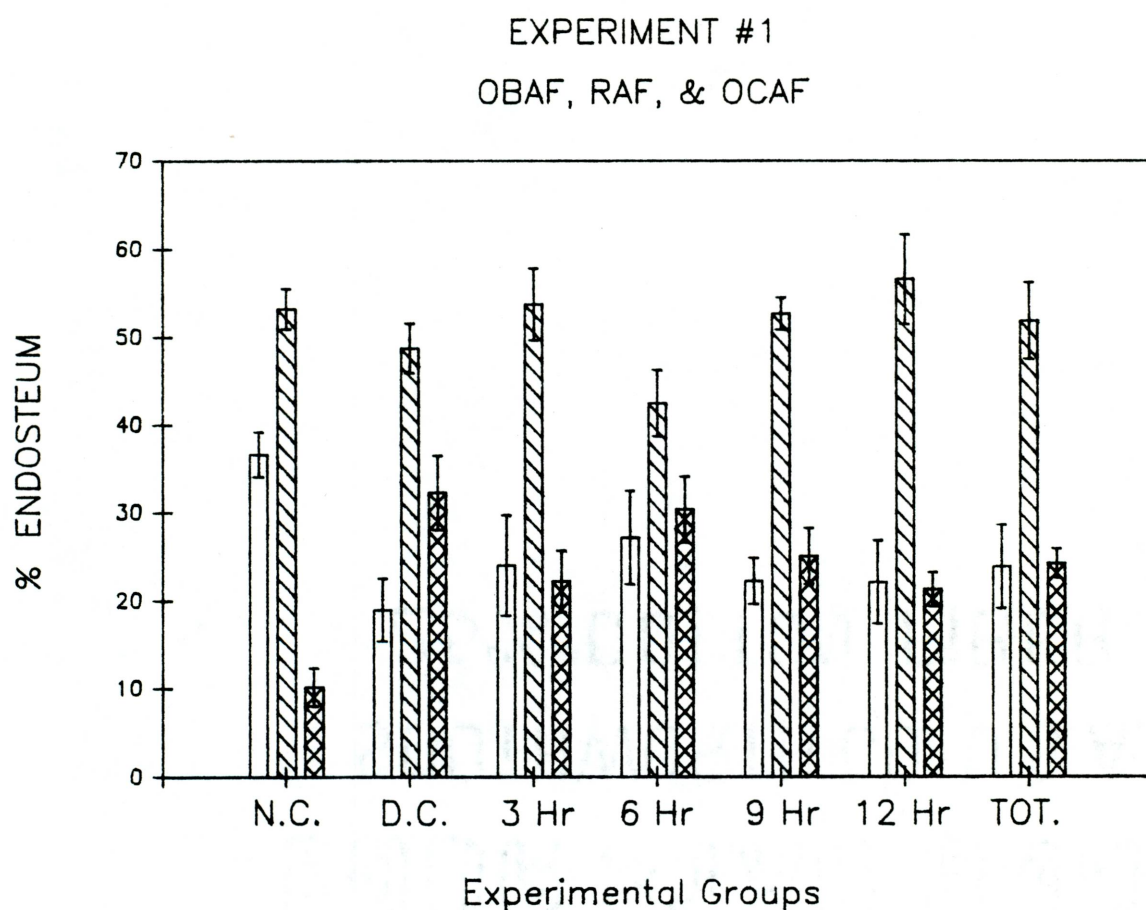


FIGURE 1: Osteoblast Area Fractions (white bars), Resting Area Fractions (diagonally striped bars), and Osteoclast Area Fractions (crosshatched bars), means \pm SEM. **Experimental groups:** N.C. = normal control, D.C. = deficient control, 3 Hr = 3-hour supplemented, etc., TOT. = total supplemented (see text). $n = 5$ for the 3-hour supplemented group; $n = 6$ for all other groups.

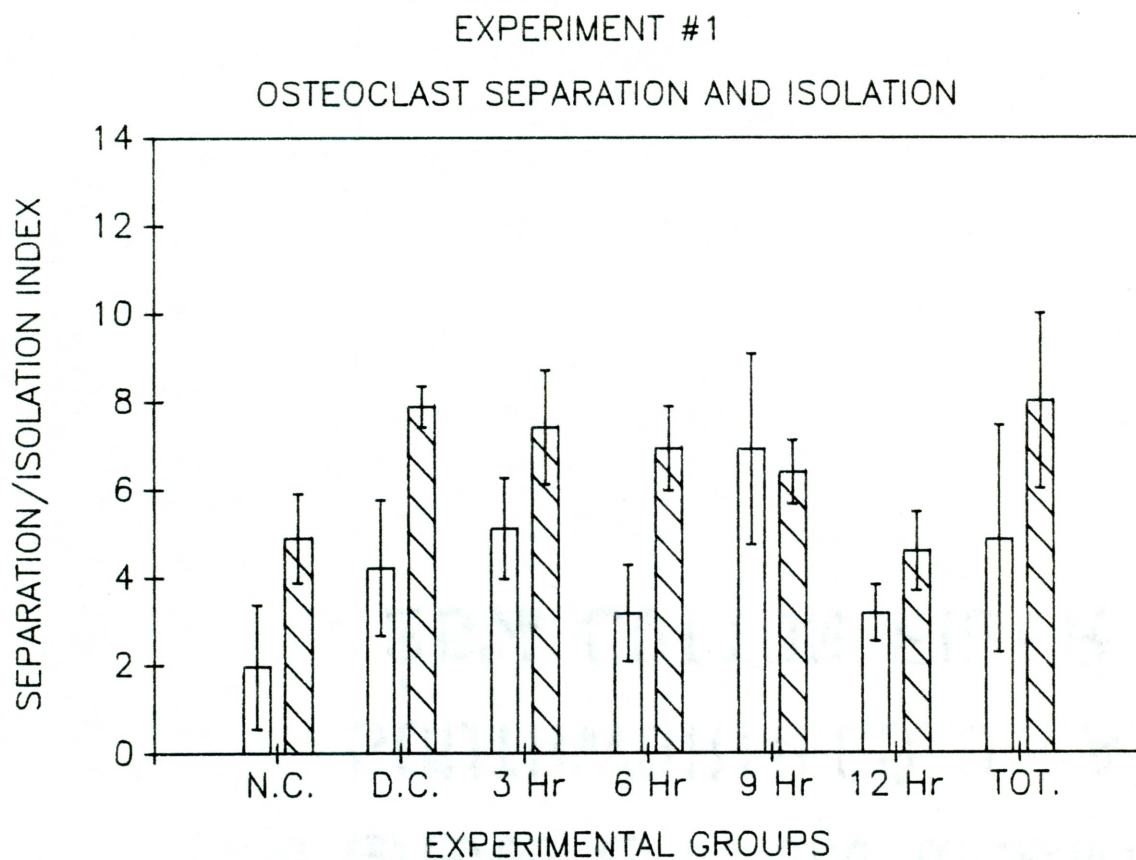


FIGURE 2: Separated Index (white bars) and Isolation Index (striped bars), means \pm SEM. **Experimental groups:** N.C. = normal control, D.C. = deficient control, 3 Hr = 3-hour supplemented, etc., TOT. = total supplemented (see text). $n = 5$ for the 3-hour supplemented group; $n = 6$ for all other groups.

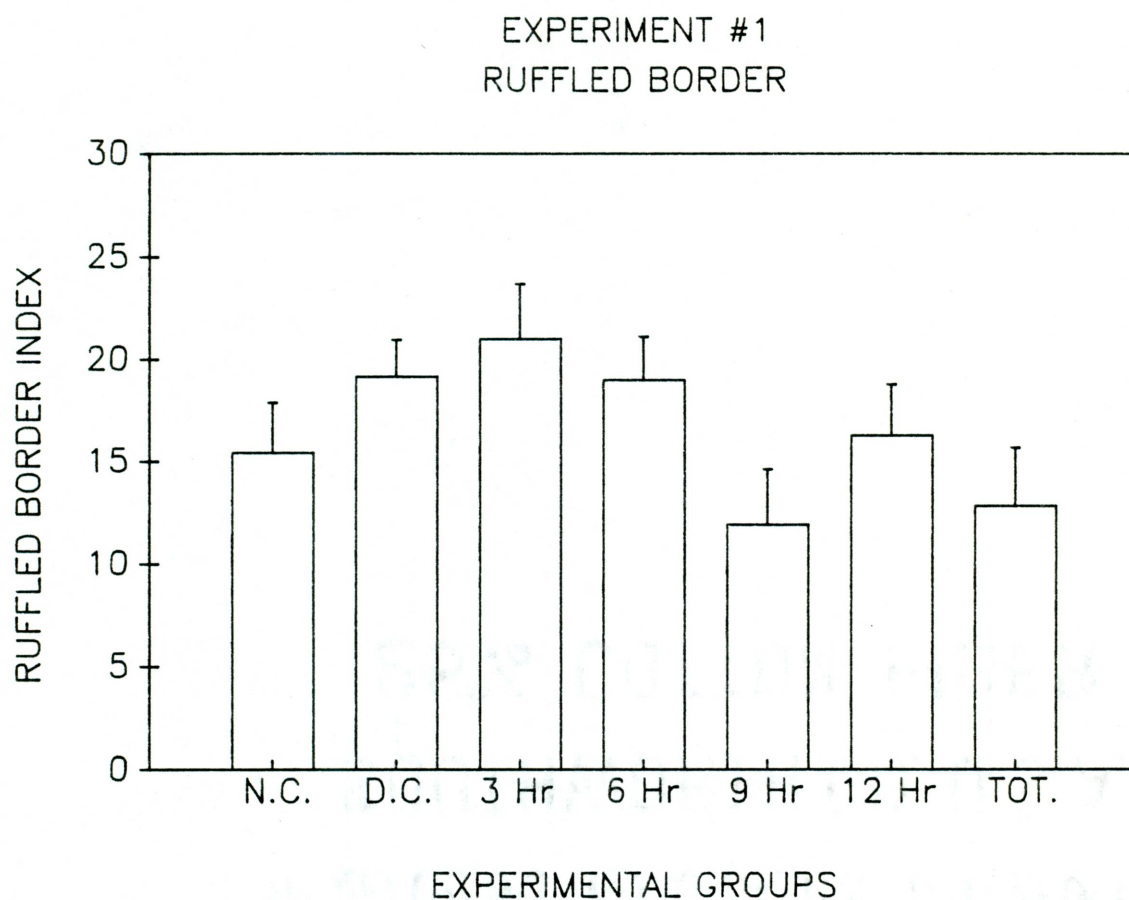


FIGURE 3: Ruffled Border Index, means \pm SEM. **Experimental groups:** N.C. = normal control, D.C. = deficient control, 3 Hr = 3-hour supplemented, etc., TOT. = total supplemented (see text). $n = 5$ for the 3-hour supplemented group; $n = 6$ for all other groups.

Of the eight morphological indices measured, none showed a significant change with increasing calcium supplementation. However, the trend toward a reduction of ruffled border with increasing calcium supplementation suggested that there was an increasing inhibition of osteoclast activity with increasing administration time of calcium.

The feeding protocol which was followed on the eighth day of the experiment called for a twelve-hour fasting period at the end of the calcium feeding period, before killing the animals, in order to use a total 24-hour time frame so that the results of this experiment could be compared with those of Liu et al. (1982). In analyzing the data, the conclusion was reached that it was because of this fasting period that the morphological indices did not show significant differences between groups. It was hypothesized that the fast lowered the blood calcium levels sufficiently to stimulate osteoclast reactivation, resulting in a lack of statistical differences between groups in osteoclast morphology indicating osteoclast inhibition.

A discrepancy should be noted between the results of this experiment and the subsequent experiments. In this first experiment, it was found that the contact index was greater in the normal than in the deficient controls, while the deficient controls showed a greater isolation index than the normal controls. No significant differences in these parameters between normal and deficient controls were found in subsequent experiments. A higher contact index suggests a greater degree of osteoclast activity, while a higher isolation index implies greater osteoclast

inhibition or greater osteoclast mobility. The results of experiment #1 could be an indication that the osteoclasts that were present in the normal controls were less mobile than those in the deficient group. Mobility could serve as an indicator for the degree of activation of the osteoclast. However, since results from subsequent experiments do not confirm these results, the significance of the data of this first experiment must be called into question.

It is interesting to note that the notion of resorption-formation coupling is not well supported by the present data. Based on the coupling theory, one would expect that when bone resorption increases, bone formation would have to increase in order to maintain an equilibrium between the two phenomena. In the calcium-deficient animal, there is a significant increase in the osteoclast area fraction, which is an indicator of increased resorption. However, there is a decrease in the osteoblast area fraction, suggesting a reduction in bone formation. The resting area fraction is comparable for the two groups. One would think that the osteoblast area fraction would have increased with the osteoclast area fraction in the deficient animals, with a decrease in the resting fraction, but this is not seen. This could be explained, perhaps, either by the severity of the deficiency making it impossible for bone formation to keep pace with resorption, or by a lag time in the formation process.

Experiment #2: Fasting Can Reverse Osteoclast Recession

The results of the first experiment led to the hypothesis that a 12-hour fast is sufficient to reverse the morphological changes toward recession manifested by

osteoclasts after twelve hours of calcium supplementation. It was therefore necessary to design an experiment which would compare two calcium-supplemented groups, one which would fast for 12 hours following the calcium supplementation, and the other which would be killed immediately at the end of the calcium supplementation period.

Four groups of six animals were used, with reversed lighting (lights on 20:30, lights off 8:30), and a habituation period of 6 days. The normal and deficient control groups received only normal calcium diet or calcium free diet during the entire experiment. The non-fasting experimental group received calcium free diet 7 days, and on day 8 received 6 grams of normal calcium diet spread over four feedings. These first three groups were killed immediately after the last feeding period. The last group, the fasted experimental group, was fed the calcium free diet for 7 days, and on day 8 received 6 grams of normal calcium diet spread over four feedings, but these animals fasted 12 hours before being killed.

Table 2 (page 50) summarizes the results of experiment #2. The two control groups, when compared by t-test, differed significantly in OBAF (normal > deficient; $p < 0.05$) and OCAF (deficient > normal; $p < 0.001$) (see Figure 4, page 51). The supplemented non-fasting group differed significantly from the deficient controls in RAF, OCAF, RBI (Figure 5, page 52), CI (Figure 6, page 53), SI (Figure 7, page 54), and II (Figure 8, page 55). Of these six indices, only RAF remained significantly elevated in the fasting group when compared to the deficient control group.

GROUP	OBAF	RAF	OCAF	RBI	CI	SI	II
N.C.	39.4 \pm 13.0	54.2 \pm 12.3	6.9 \pm 3.5	17.1 \pm 14.9	82.4 \pm 11.7	4.9 \pm 6.8	12.8 \pm 6.8
D.C.	24.1 \pm 10.1	48.5 \pm 6.5	28.4 \pm 10.6	23.4 \pm 7.6	84.5 \pm 4.1	4.0 \pm 3.3	11.5 \pm 1.8
FAST	18.0 \pm 9.8	62.1 \pm 10.0	21.0 \pm 5.6	18.8 \pm 5.3	84.3 \pm 4.1	4.7 \pm 2.5	11.0 \pm 3.4
NO FAST	25.3 \pm 5.0	61.8 \pm 6.4	15.0 \pm 3.9	8.5 \pm 2.4	63.6 \pm 5.9	14.3 \pm 4.4	22.1 \pm 4.8

TABLE 2: Data for Experiment #2, means \pm SD

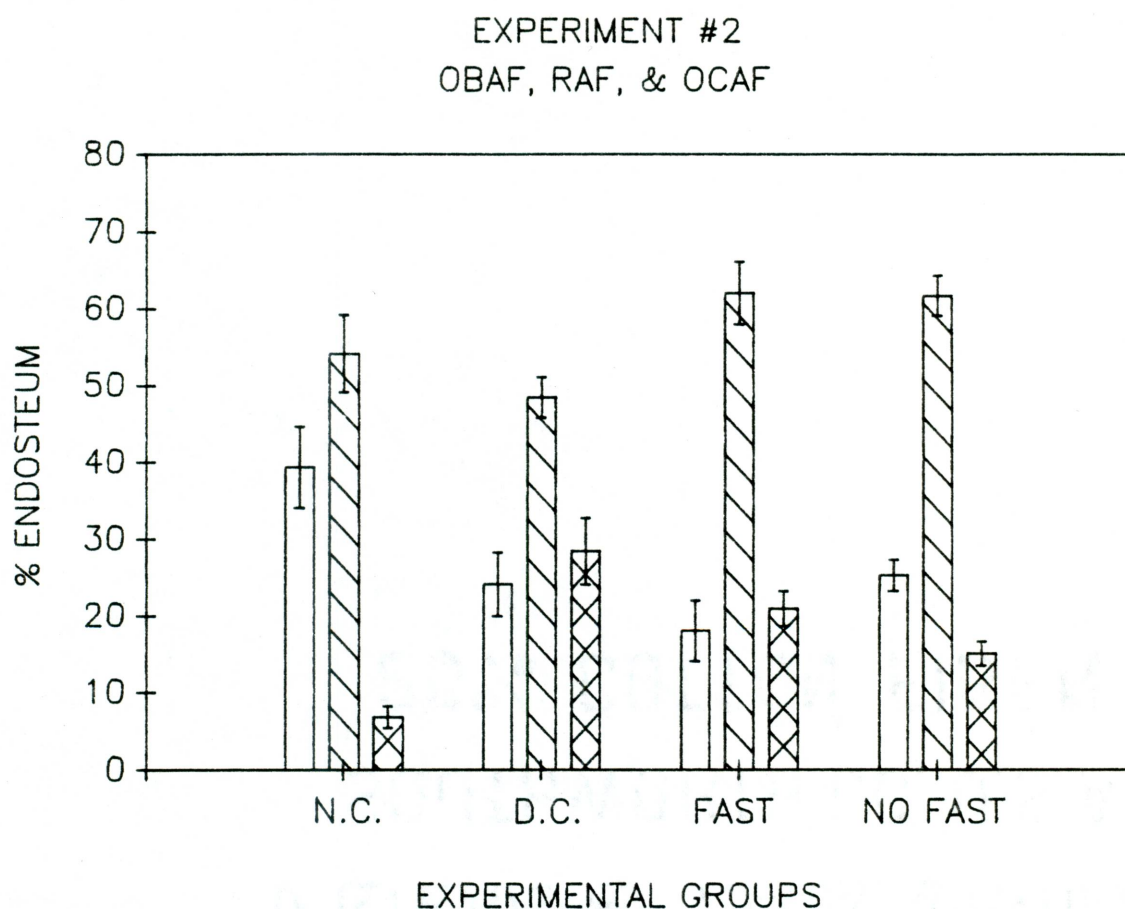


FIGURE 4: Osteoblast Area Fraction (white bars), Resting Area Fraction (striped bars), and Osteoclast Area Fraction (crosshatched bars), means \pm SEM. **Experimental groups:** N.C. = normal control, D.C. = deficient control, FAST = fasted supplemented, NO FAST = non-fasted supplemented. $n = 6$ for all groups.

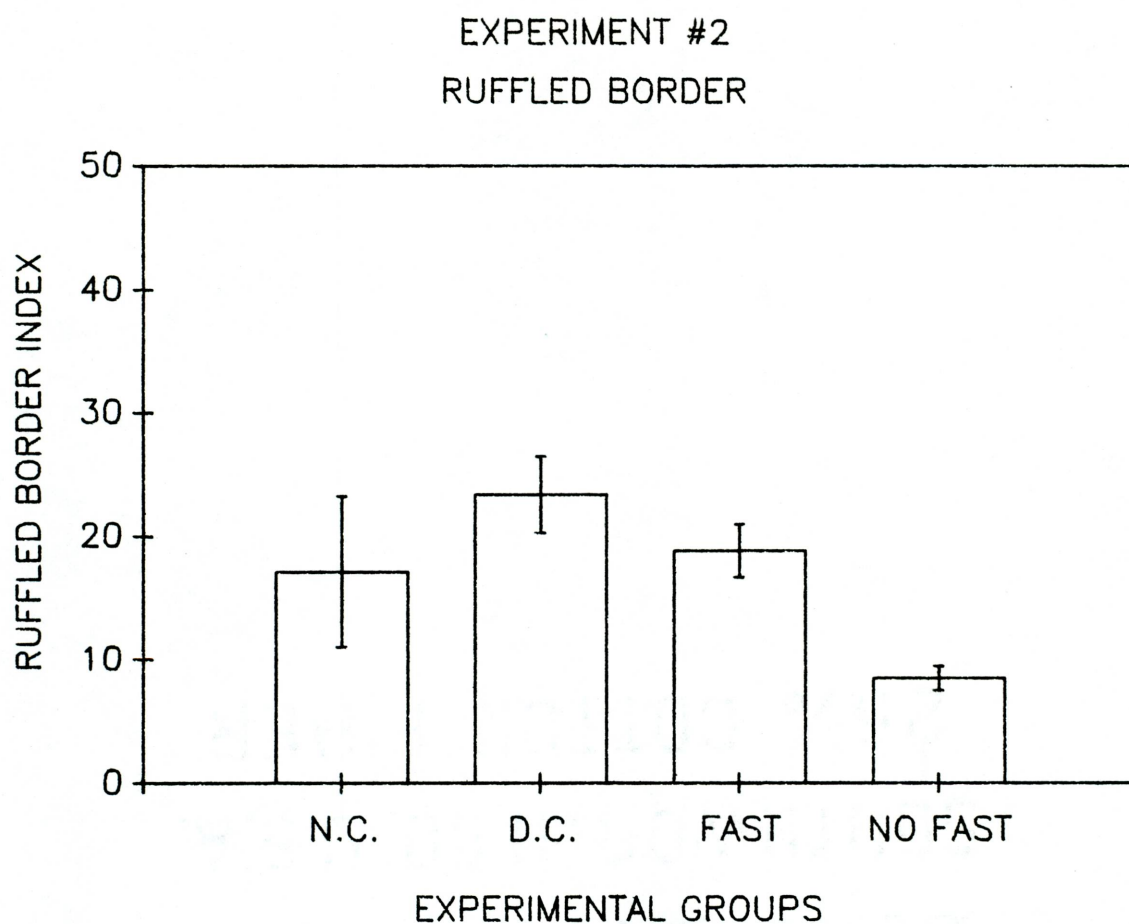


FIGURE 5: Ruffled Border Index, means \pm SEM. **Experimental groups:** N.C. = normal control, D.C. = deficient control, FAST = fasted supplemented, NO FAST = non-fasted supplemented. $n = 6$ for all groups.

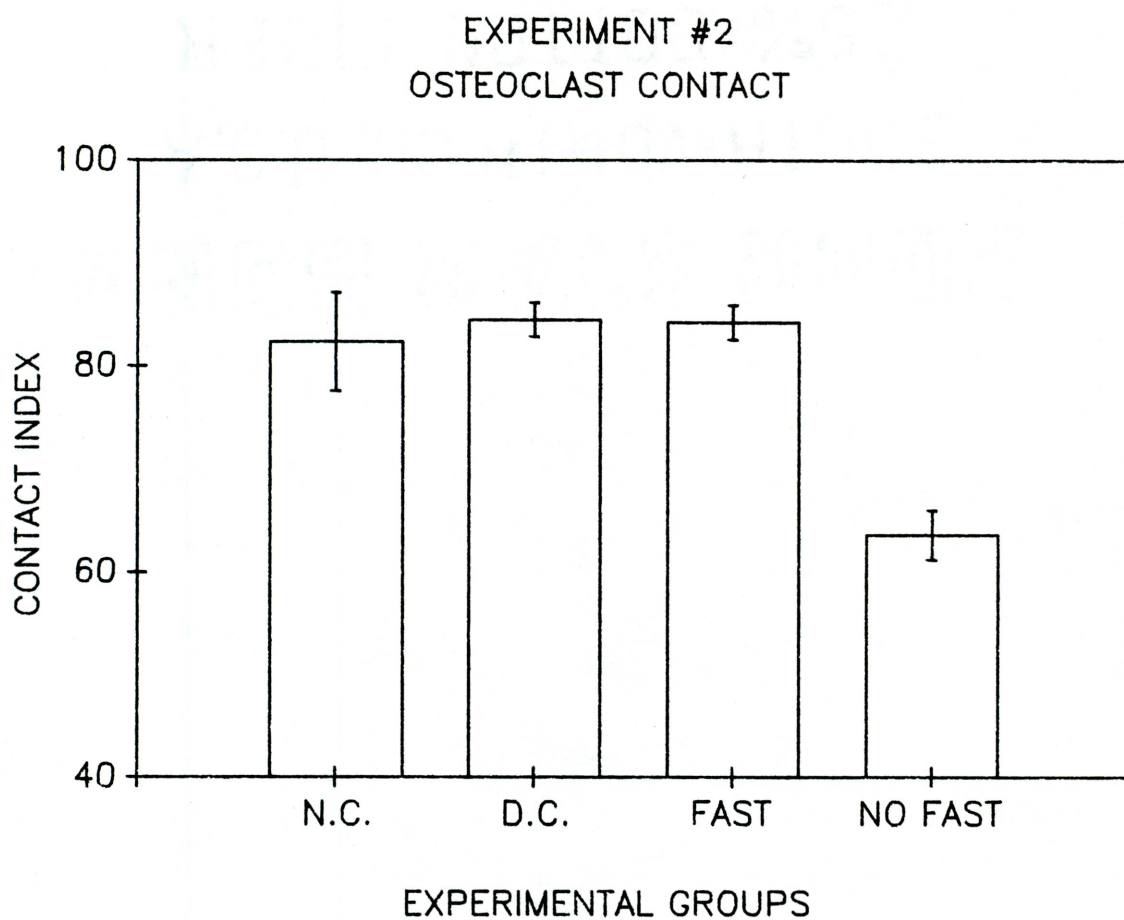


FIGURE 6: Osteoclast Contact Index, means \pm SEM. **Experimental groups:**
N.C. = normal control, D.C. = deficient control, FAST = fasted supplemented, NO FAST = non-fasted supplemented. $n = 6$ for all groups.

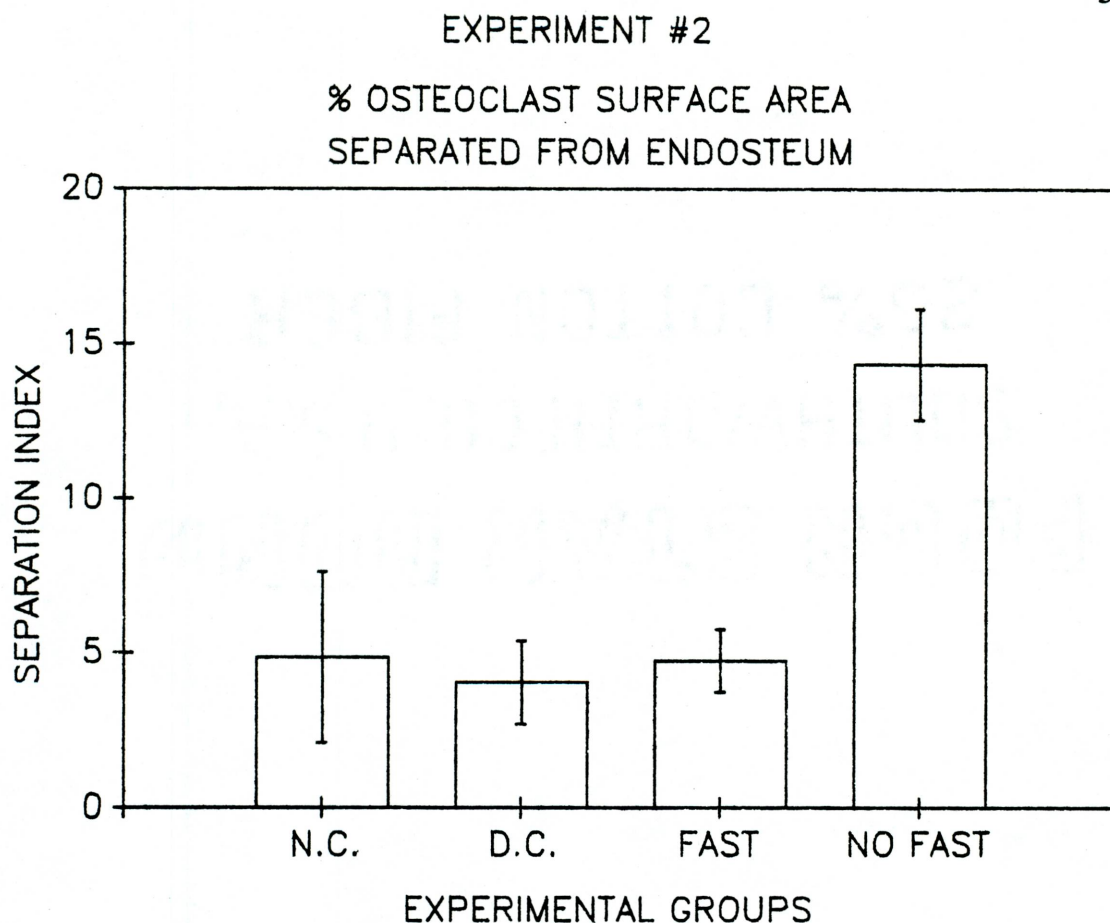


FIGURE 7: Osteoclast Separation Index, means \pm SEM. **Experimental groups:** N.C. = normal control, D.C. = deficient control, FAST = fasted supplemented, NO FAST = non-fasted supplemented. $n = 6$ for all groups.

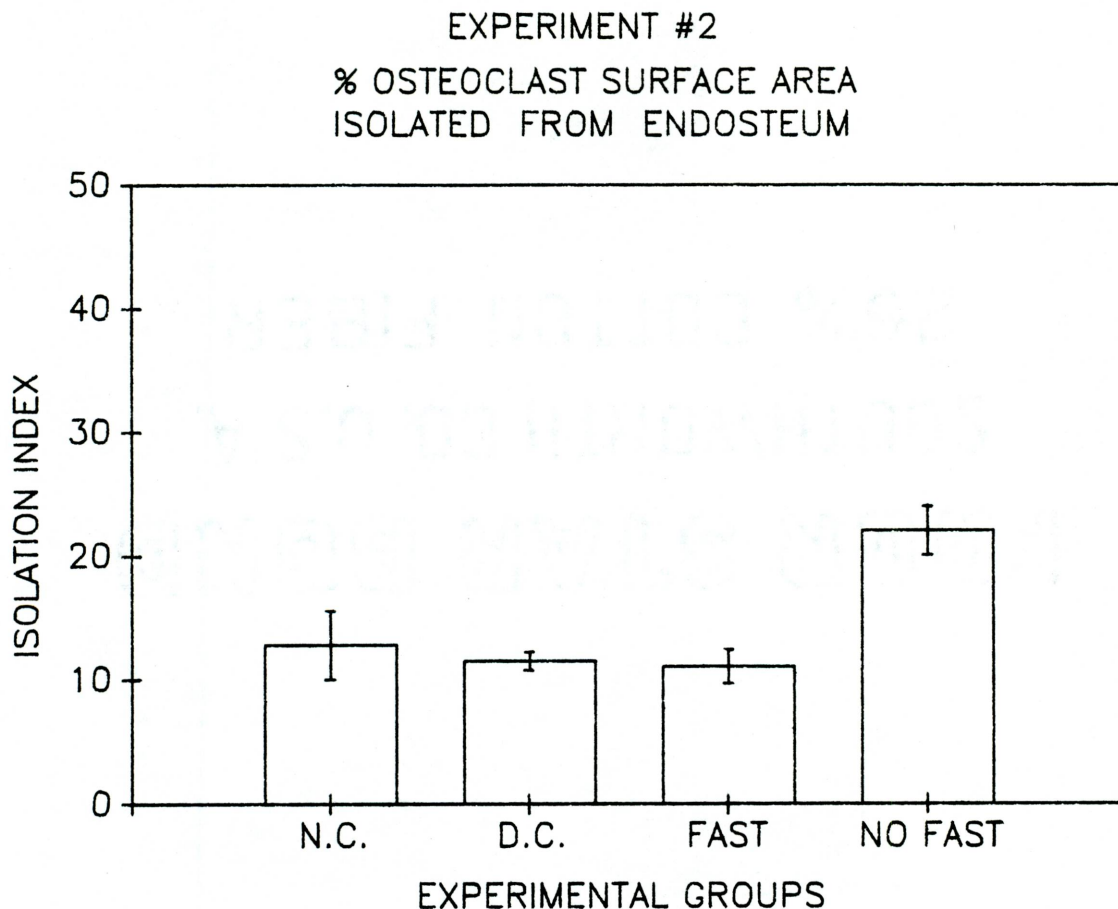


FIGURE 8: Osteoclast Isolation Index, means \pm SEM. **Experimental groups:**
N.C. = normal control, D.C. = deficient control, FAST = fasted
supplemented, NO FAST = non-fasted supplemented. $n = 6$ for all groups.

The results indicate that the 12 hours of calcium supplementation is sufficient to cause morphological changes in osteoclasts that are indicative of inhibition. The supplemented group had a decreased OCAF and an increased RAF compared to the deficient control, suggesting that a decrease in osteoclast activity due to calcium-induced inhibition is balanced by an increase in the resting cell fraction, an indication that while osteoclasts are rapidly shut down, osteoblastic activity is not immediately increased.

The indices of osteoclast activity, namely the ruffled border index and the contact index, are both decreased in the non-fasting supplemented group compared to the deficient control animals, while indices of osteoclast inhibition, the osteoclast separation and isolation indices, have both increased in the non-fasting supplemented animals as compared to the deficient controls. Both of these facts support the idea that 12 hours of calcium supplementation in calcium-deficient animals is sufficient to initiate osteoclast recession. In this and in all subsequent studies, the removed index failed to show significant differences between groups, and so will not be discussed further. This apparent lack of statistical differences was attributed to the difficulty in identifying osteoclasts or osteoclast fragments morphologically, without the use of specific cytoplasmic markers, once the osteoclasts have left the bone surface.

Whereas the non-fasting supplemented group shows very significant morphological differences from the deficient controls, the fasting group is difficult to distinguish from the deficient animals. Only the RAF remains significantly

higher in the fasting supplemented animals than in the deficient animals ($p < 0.01$), suggesting that the resting population might act as a sort of buffer when rapid changes take place at the endosteum. The other morphological indices have all returned to deficient control values, indicating that the fast is effective in reactivating osteoclasts that have been at least partially inhibited by calcium supplementation.

**Experiment #3: Graded Response of Osteoclasts Demonstrated by
Comparing Restricted to ad libitum Calcium
Supplementation**

In *experiment #2*, weanling rats were given a calcium-deficient diet for 7 days followed by a calcium-containing diet for 12 hours, and then immediately killed. The feeding protocol on day 8 consisted of 4 feedings of 1.5 grams each, at 3-hour intervals, for a total of 6 grams over twelve hours. This feeding schedule was used because it was felt that in order to be sure all animals ate the same amount of food, and attained similar serum calcium levels, both the amount of food and time frame of the feedings needed to be controlled. The reason for giving 6 grams of diet was that in a previous experiment it was found that the rats consumed an average of 7 grams of diet per 12-hour feeding period. It was thought that if given slightly less than the 7-gram average, the animals would be likely to consume all food presented to them.

In preparation for *experiment #2*, the diet for the special feeding to be carried out on day 8 was weighed out into 1.5 gram aliquots and sealed in plastic pouches before the experiment began, so that the feeding could be carried out

with the greatest possible ease and efficiency. In monitoring the consumption of food during the 7-day deficiency period, when animals were allowed to eat to satiation during each daily 12-hour feeding period, it was found that these animals were consuming an average of 10 to 13 grams per 12-hour period, as opposed to the 7 grams that had previously been observed. This led to the question of whether the restricted supplementation would put the experimental animals in a semi-fasting state. A hypothesis was proposed stating that the restricted calcium supplementation regime given on day eight would cause a smaller change in osteoclast morphology than an *ad libitum* supplementation. In this third experiment, comparison was made between the restricted feeding schedule used in experiment #2 and *ad libitum* feeding. The hypothesis that 24 hours of *ad libitum* calcium supplementation would bring about greater morphological changes in osteoclasts and other endosteal cells than 12 hours of calcium supplementation was also tested.

Three groups of 6 rats were used, again with reversed lighting and a six-day habituation period. All three groups received calcium free diet for 7 days. On day 8, the first group received four feedings of 1.5 g of normal calcium diet per feeding, at three-hour intervals, over a 12-hour period as in experiment #2. Group 2 received normal calcium diet *ad libitum* for 12 hours. Group 3 received 24 hours normal calcium diet *ad libitum*. Each group was killed at the end of the supplemented feeding period.

See table 3 (page 60) for a summary of the results of experiment #3.

Significant differences were found between the restricted and 12-hour *ad libitum* groups in RAF (*ad libitum* > restricted; $p=0.01$)(Figure 9, page 61), OCAF (restricted > *ad libitum*; $p=0.001$)(Figure 9, page 61), RBI (restricted > *ad libitum*; $p=0.002$)(Figure 10, page 62), CI (restricted > *ad libitum*; $p=0.03$)(Figure 11, page 63) and II (*ad libitum* > restricted; $p=0.03$)(Figure 12, page 64). Some differences were also found between the 12-hour and 24-hour *ad libitum* feeding groups: OBAF (24-hour > 12-hour; $p=0.03$)(Figure 9, page 61) and OCAF (12-hour > 24-hour; $p=0.03$)(Figure 9, page 61). The OCAF was also significantly greater in the restricted group than in the 24-hour *ad libitum* group ($p<0.0001$)(Figure 9, page 61).

The 12-hour *ad libitum* group was more effective in bringing about osteoclast recession than the restricted supplemented group, since the OCAF was significantly lower in the *ad libitum* group. Again, the resting cell fraction seemed to compensate for the decrease in osteoclast numbers, while the osteoblast fraction did not change. A decrease in the ruffled border and the contact indices, and an increase in the separation and isolation indices, also demonstrated the effectiveness of the *ad libitum* feeding protocol in inducing osteoclast recession compared to the restricted regime.

Interestingly, after 24 hours of *ad libitum* feeding, the osteoblast area fraction began to increase, suggesting an increase in osteoblastic activity, as the osteoclast area fraction continued to decrease. This suggests two things: first, that osteoclast

GROUP	OBAF	RAF	OCAF	RBI	CI	SI	II
Res.	24.1 \pm 5.9	56.7 \pm 4.9	19.8 \pm 4.6	13.7 \pm 4.9	78.9 \pm 5.6	3.0 \pm 1.7	18.1 \pm 6.3
12 ad	21.2 \pm 11.4	67.5 \pm 8.9	11.8 \pm 3.2	3.3 \pm 1.7	67.2 \pm 5.3	4.4 \pm 2.3	28.4 \pm 4.6
24 ad	32.4 \pm 4.9	60.6 \pm 4.0	7.3 \pm 1.6	8.5 \pm 6.0	71.1 \pm 12.3	2.9 \pm 3.1	26.0 \pm 9.7

TABLE 3: Data for Experiment #3, means \pm SD

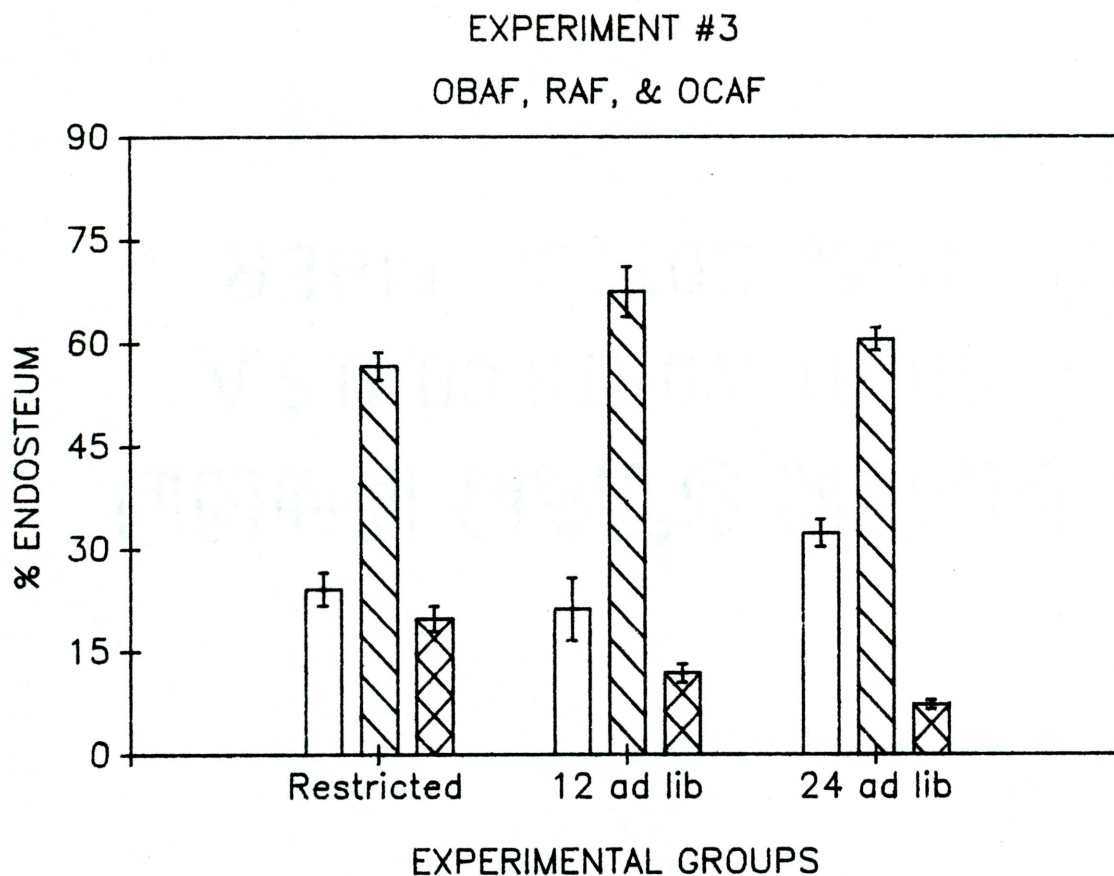


FIGURE 9: Osteoblast Area Fraction (white bars), Resting Area Fraction (striped bars), and Osteoclast Area Fraction (crosshatched bars), means \pm SEM. **Experimental groups:** Restricted = restricted supplemented, 12 ad lib = 12-hour *ad lib* supplemented, 24 ad lib = 24-hour *ad lib* supplemented. $n = 6$ for all groups.

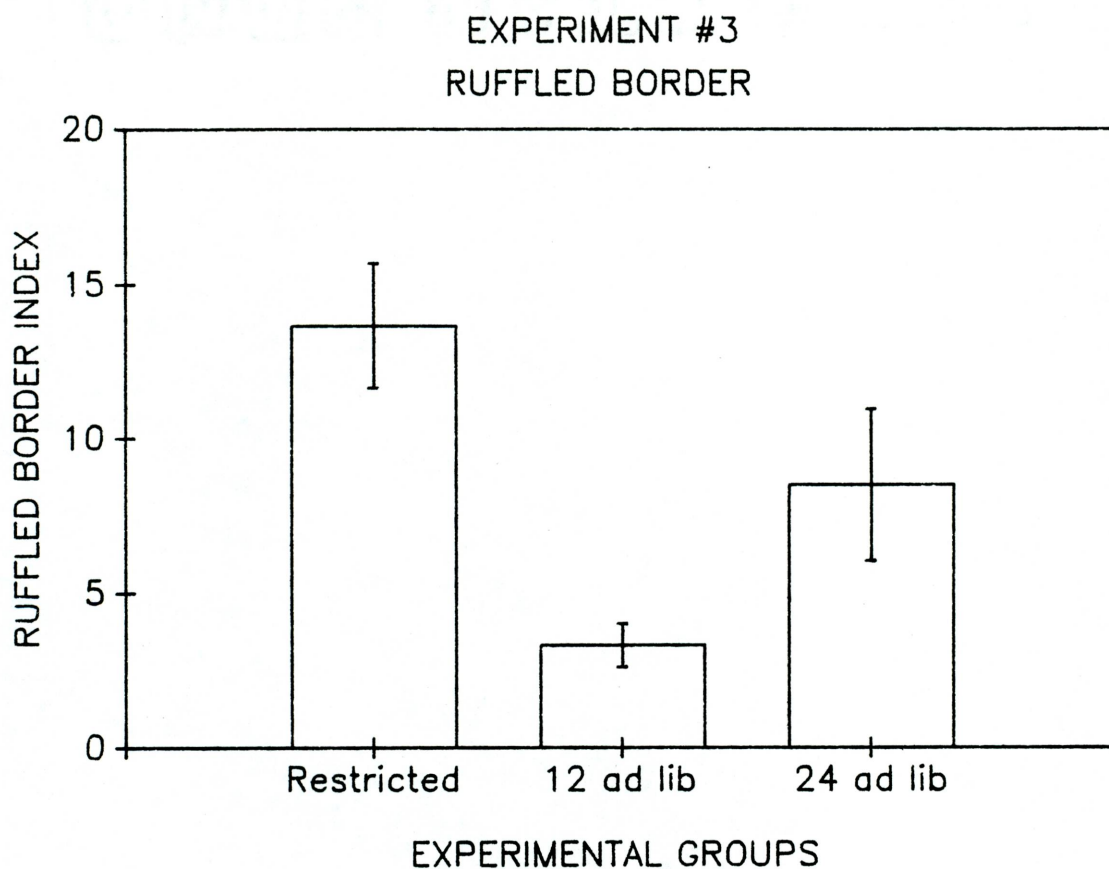


FIGURE 10: Ruffled Border Index, means \pm SEM. **Experimental groups:** Restricted = restricted supplemented, 12 ad lib = 12-hour *ad lib* supplemented, 24 ad lib = 24-hour *ad lib* supplemented. $n = 6$ for all groups.

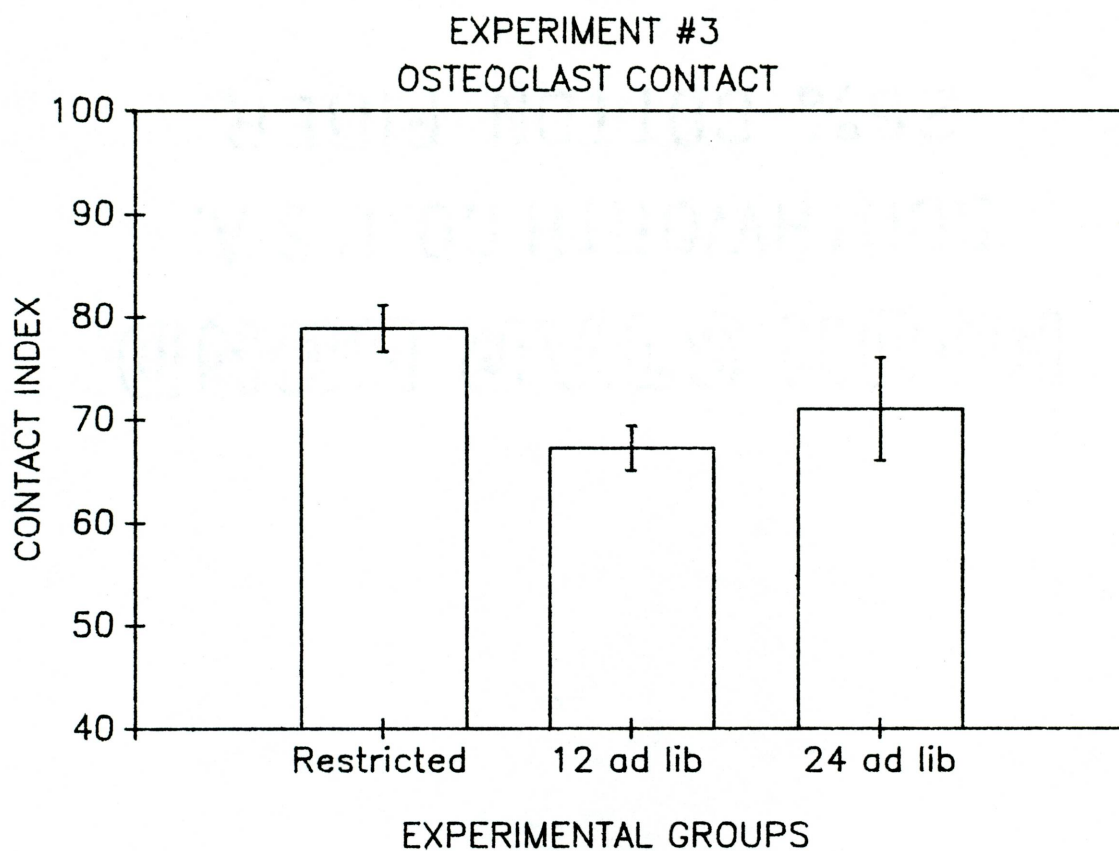


FIGURE 11: Osteoclast Contact Index, means \pm SEM. **Experimental groups:**
Restricted = restricted supplemented, 12 ad lib = 12-hour *ad lib* supplemented, 24 ad lib = 24-hour *ad lib* supplemented.
n = 6 for all groups.

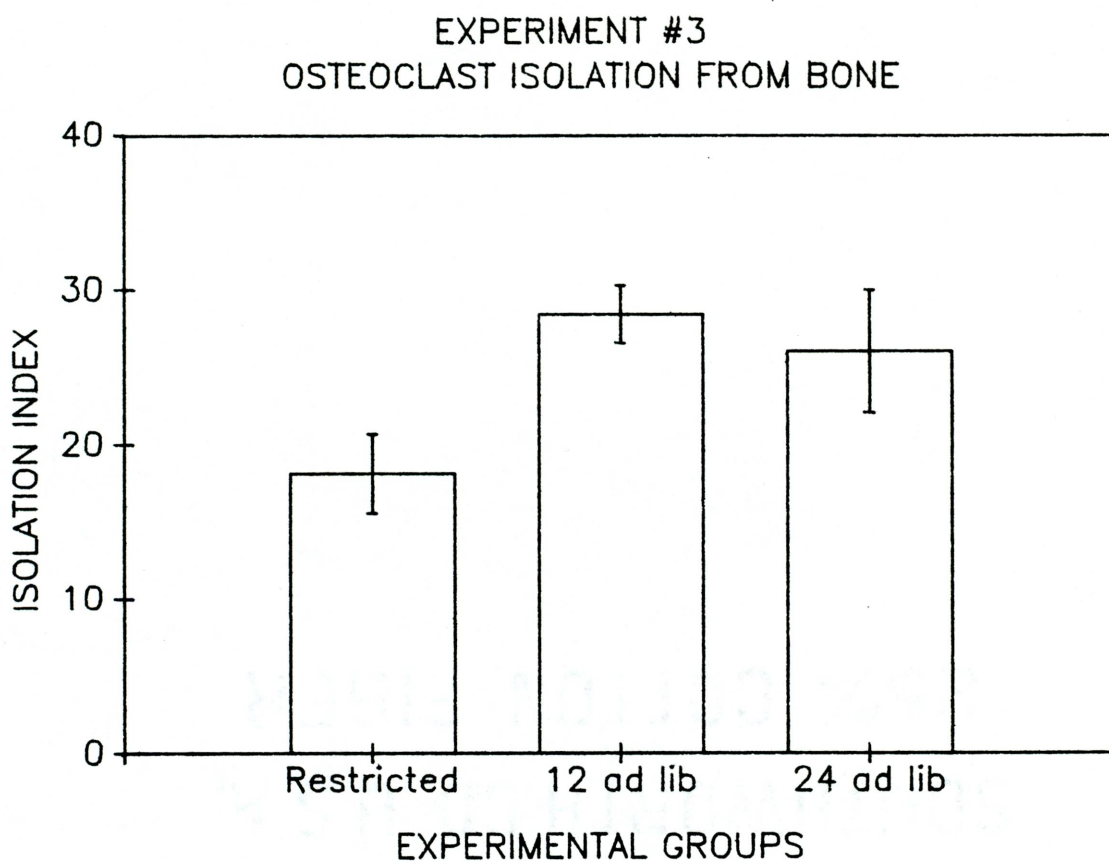


FIGURE 12: Osteoclast Isolation Index, means \pm SEM. **Experimental groups:** Restricted = restricted supplemented, 12 ad lib = 12-hour *ad lib* supplemented, 24 ad lib = 24-hour *ad lib* supplemented. $n = 6$ for all groups.

inhibition is more rapid than osteoblast stimulation; and second, that the rats must have continued eating a significant amount of food even during the light part of the light/dark cycle in order for the OCAF to have continued to decrease, since a fast would have tended to increase the OCAF.

As a result of this experiment, it was decided to feed the animals *ad libitum* and to leave the food in the cage during both the light and dark periods of each daily cycle in subsequent experiments.

Summary of Part 1

McMillan's work had shown that changes in osteoclast morphology indicating a movement toward recession could be detected after 3 hours on a calcium-containing diet. It was thought that increasing the amount of calcium supplementation would increase osteoclast recession until a point of total removal from bone had been achieved. However, the results did not bear this out. The group receiving the most calcium supplementation showed a trend toward decreased RBI but this was not statistically significant. These results were attributed to the 12-hour fast.

The next experiment showed that the fast did in fact reverse the changes in osteoclast morphology brought about by the calcium supplementation. And the third experiment showed that *ad libitum* feeding of the normal calcium diet was more effective in bringing about osteoclast recession than the restricted regime that had been used in previous experiments. This evidence supported the hypothesis

that osteoclast inhibition is a graded phenomenon, responding, in part, to the amount of calcium given after the period of calcium deficiency.

These three experiments indicated that short-term fasting could reactivate inhibited osteoclasts. The question arose whether longer-term fasting could be a means of initially recruiting osteoclasts in normal animals. The second part of this study compared the effects of fasting and calcium deficiency on osteoclast recruitment and on changes in endosteal cell populations.

Osteoclast Recruitment by Fasting and Calcium Deficiency

Experiment #4: Fasting vs Calcium deficiency as a Means of Osteoclast Activation

Having shown that fasting could reactivate osteoclasts that had begun receding from the bone surface, it was necessary to examine and compare fasting and calcium-deficient diet as ways of initially recruiting and activating osteoclasts, in order to see if the same principles were operating in both situations, or whether the mechanisms were different.

Four groups of six rats were given calcium-containing diet for 7 days. Two groups were then fasted either 24 or 48 hours, and two groups received calcium free diet *ad libitum* for either 24 or 48 hours. All animals were killed immediately after the fasting or feeding period and tissues were prepared for morphometric analysis.

Table 4 (page 67) shows the results for experiment #4. The 48-hour fasting group had a smaller OBAF than the other three groups ($p < 0.05$), and a greater RAF than the other groups ($p < 0.001$)(Figure 13, page 68). The 48-hour calcium

GROUP	OBAF	RAF	OCAF	RBI	CI	SI	II
24 FAST	43.5 \pm 19.2	45.8 \pm 16.8	10.8 \pm 5.0	29.4 \pm 9.4	93.5 \pm 2.8	0.4 \pm 0.4	6.1 \pm 2.8
48 FAST	17.7 \pm 8.6	73.7 \pm 9.1	8.6 \pm 4.6	25.1 \pm 17.0	92.6 \pm 5.4	1.1 \pm 1.4	6.3 \pm 4.1
24 DEF.	51.0 \pm 18.9	39.1 \pm 13.8	10.1 \pm 6.5	20.1 \pm 10.1	93.6 \pm 5.4	1.5 \pm 3.2	5.0 \pm 3.5
48 DEF.	38.9 \pm 9.0	44.2 \pm 8.5	17.3 \pm 7.6	16.7 \pm 11.6	91.2 \pm 8.9	2.4 \pm 4.8	6.5 \pm 4.7

TABLE 4: Data for Experiment #4, means \pm SD

EXPERIMENT #4
OBAF, RAF, & OCAF

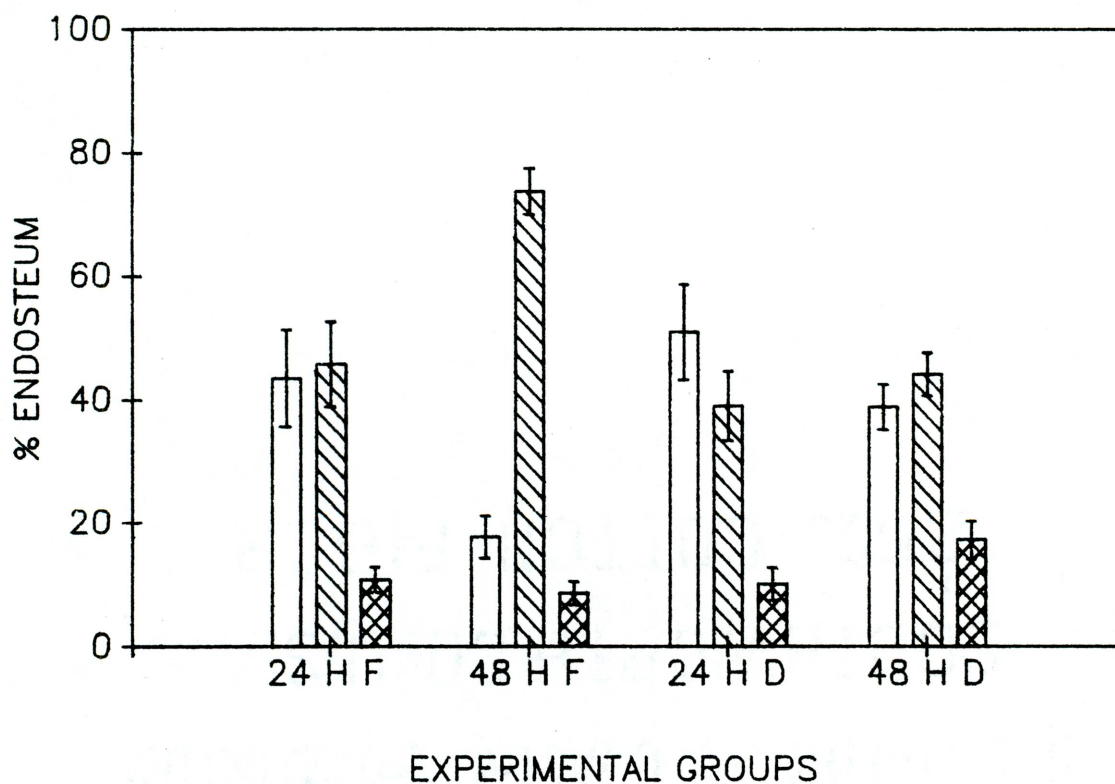


FIGURE 13: Osteoblast Area Fraction (white bars), Resting Area Fraction (striped bars), and Osteoclast Area Fraction (crosshatched bars), means \pm SEM. **Experimental groups:** 24 H F = 24-hour fasted, 48 H F = 48-hour fasted, 24 H D = 24-hour deficient, 48 H D = 48-hour deficient. $n = 6$ for all groups.

free group had a greater OCAF than the other groups, but the difference was only statistically significant between the 48-hour calcium free group and the 48-hour fasting group ($p=0.02$)(Figure 13, page 68). The other morphological indices showed no significant differences between groups. These results suggest that fasting shuts down osteoblast activity, but instead of stimulating osteoclast recruitment and activation, the resting cell fraction increases. Fasting, then, would seem to maintain serum calcium levels by stopping bone formation (but not by increasing resorption), as indicated by the decreased OBAF and the increased RAF in the 48-hour fasting group. On the other hand, the results of 48-hour of calcium deficiency, that is, an increase in the OCAF, suggest that in a calcium-deficient state, calcium homeostasis is maintained, at least short-term, by an increase in bone resorption.

Experiment #5: De novo Osteoclast Recruitment vs Re-recruitment by Calcium Deficiency

The previous experiments in this study have indicated that short-term fluctuations in dietary calcium have the effect of modulating osteoclast activity rather than bringing in new osteoclasts or removing old ones. Osteoclasts appear to be under dynamic control rather than a simple OFF/ON mechanism. While osteoclasts begin to show morphological changes rapidly with calcium supplementation, it appears that approximately 24 or more hours of calcium supplementation is required to cause recession of the entire osteoclast population from bone. The final hypothesis to be tested was that osteoclasts that had been inhibited could be re-recruited, which would be evidenced by a more rapid rate of

recruitment than an initial osteoclast recruitment. The outcome of this experiment would depend on the fate of the inhibited osteoclast.

Six groups of 6 animals were used, as defined below:

7-day deficient control: 7 days calcium free diet

9-day normal control: 9 days normal calcium diet

9-day deficient control: 9 days calcium free diet

Supplemented control: 7 days calcium free diet followed by 2 days normal calcium diet

De novo recruitment: 9 days normal calcium diet, 2 days calcium free diet

Re-recruitment: 7 days calcium free diet, 2 days normal calcium diet, 2 days calcium free diet

Animals were killed immediately after the last feeding and tissues prepared for morphometric analysis.

The results of experiment 5 are summarized in table 5 (page 71). Results of analysis of variance showed that the re-recruitment group had a significantly greater OBAF ($p < 0.01$), and a smaller RAF ($p < 0.01$) than all the other groups (Figure 14, page 72). The two calcium deficient controls had significantly greater OCAFs than the other four groups ($p < 0.005$), and the *de novo* recruitment group had a greater OCAF than the normal ($p = 0.05$) and supplemented controls ($p < 0.05$) and the re-recruitment group (Figure 14, page 72). The supplemented control had a significantly smaller RBI ($p < 0.01$)(Figure 15, page 73) and CI ($p < 0.0001$)(Figure 16, page 74) and a greater SI ($p < 0.05$)(Figure 17, page 75) and II ($p < 0.0001$)(Figure 18, page 76) than all the other groups. Another parameter, the average osteoclast profile width, was measured in this study.

GROUP	OBAF	RAF	OCAF	RBI	CI	SI	II	PROFILE
7 D.C.	36.2 \pm 8.8	40.7 \pm 8.0	23.1 \pm 5.7	27.4 \pm 7.5	90.7 \pm 4.1	1.9 \pm 1.1	7.4 \pm 3.1	9.3 \pm 0.5
9 N.C.	48.7 \pm 16.2	45.9 \pm 15.9	5.4 \pm 3.4	15.7 \pm 9.4	89.2 \pm 8.3	3.4 \pm 2.6	7.4 \pm 6.0	5.9 \pm 1.2
9 D.C.	37.9 \pm 9.6	41.7 \pm 6.4	20.3 \pm 6.3	26.3 \pm 7.0	90.1 \pm 2.6	2.3 \pm 1.1	7.7 \pm 2.0	9.2 \pm 0.5
S.C.	46.7 \pm 12.3	49.6 \pm 12.9	3.8 \pm 1.6	3.8 \pm 4.6	63.0 \pm 15.1	8.9 \pm 10.6	28.1 \pm 9.9	6.8 \pm 0.4
NOVO	40.6 \pm 13.7	48.3 \pm 15.3	11.2 \pm 7.6	22.5 \pm 10.1	89.2 \pm 10.2	2.5 \pm 3.0	8.4 \pm 7.4	8.0 \pm 0.7
RE-R	68.5 \pm 7.7	23.5 \pm 6.3	8.1 \pm 2.9	29.0 \pm 5.2	89.8 \pm 6.1	2.8 \pm 3.0	8.6 \pm 4.7	8.5 \pm 0.7

TABLE 5: Data for Experiment #5, means \pm SD

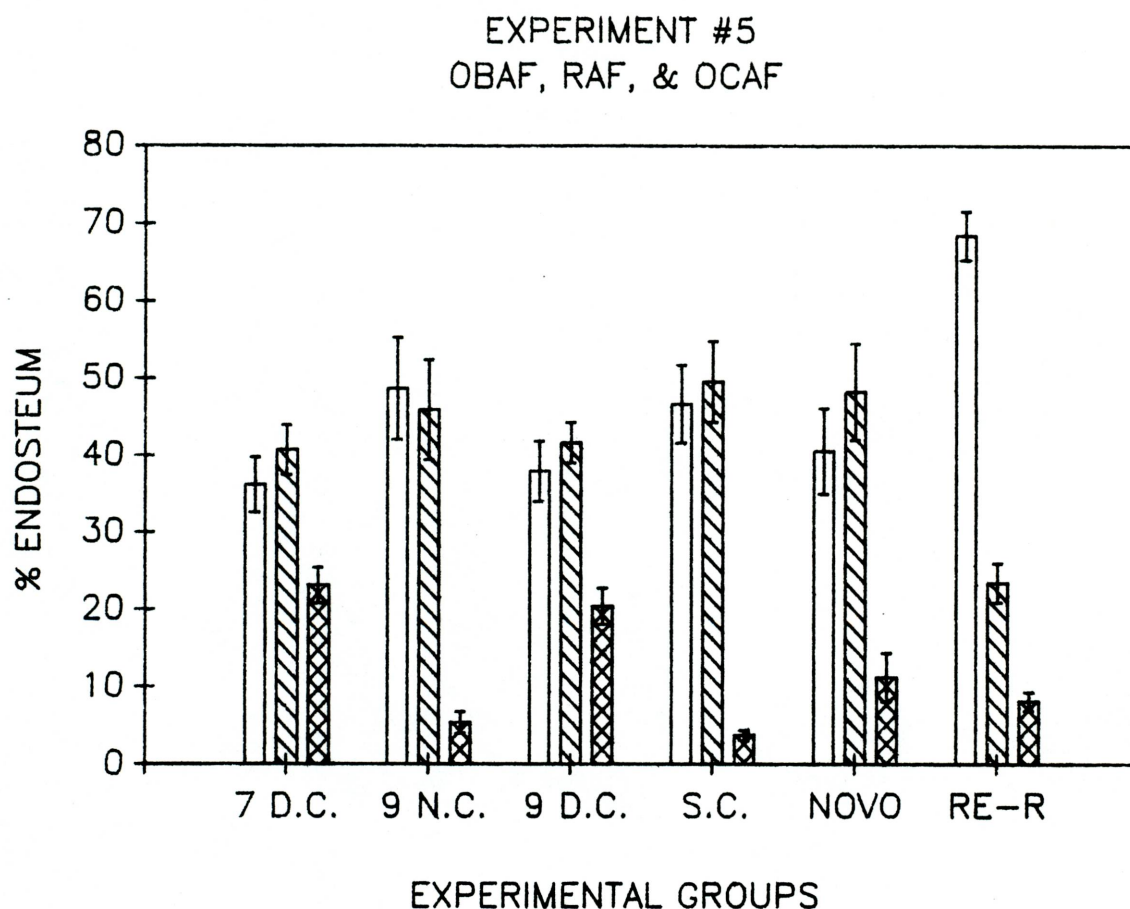


FIGURE 14: Osteoblast Area Fraction (white bars), Resting Area Fraction (striped bars), and Osteoclast Area Fraction (crosshatched bars), means \pm SEM. **Experimental groups:** 7 D.C. = 7-day deficient control, 9 N.C. = 9-day normal control, 9 D.C. = 9-day deficient control, S.C. = supplemented control, NOVO = *de novo* recruitment, RE-R = re-recruitment. $n = 6$ for all groups.

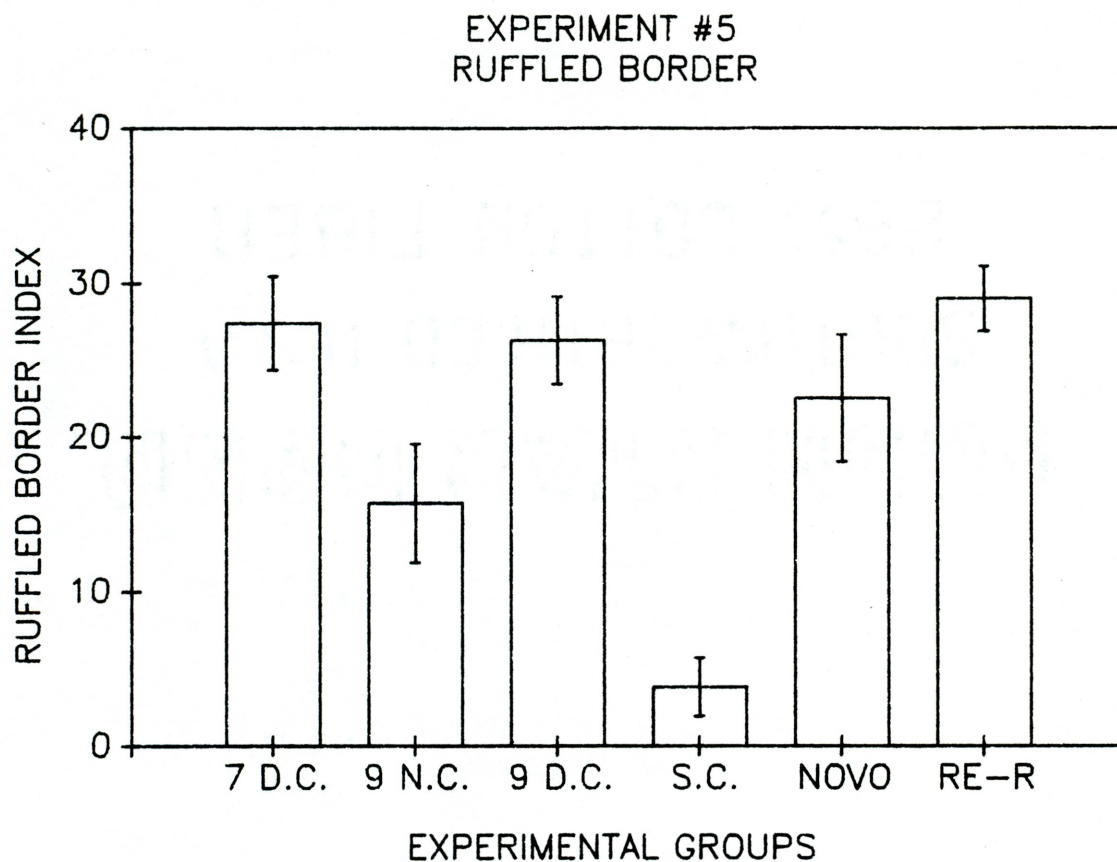


FIGURE 15: Ruffled Border Index, means \pm SEM. **Experimental groups:**
7 D.C. = 7-day deficient control, 9 N.C. = 9-day normal control,
9 D.C. = 9-day deficient control, S.C. = supplemented control,
NOVO = *de novo* recruitment, RE-R = re-recruitment. $n = 6$ for all groups.

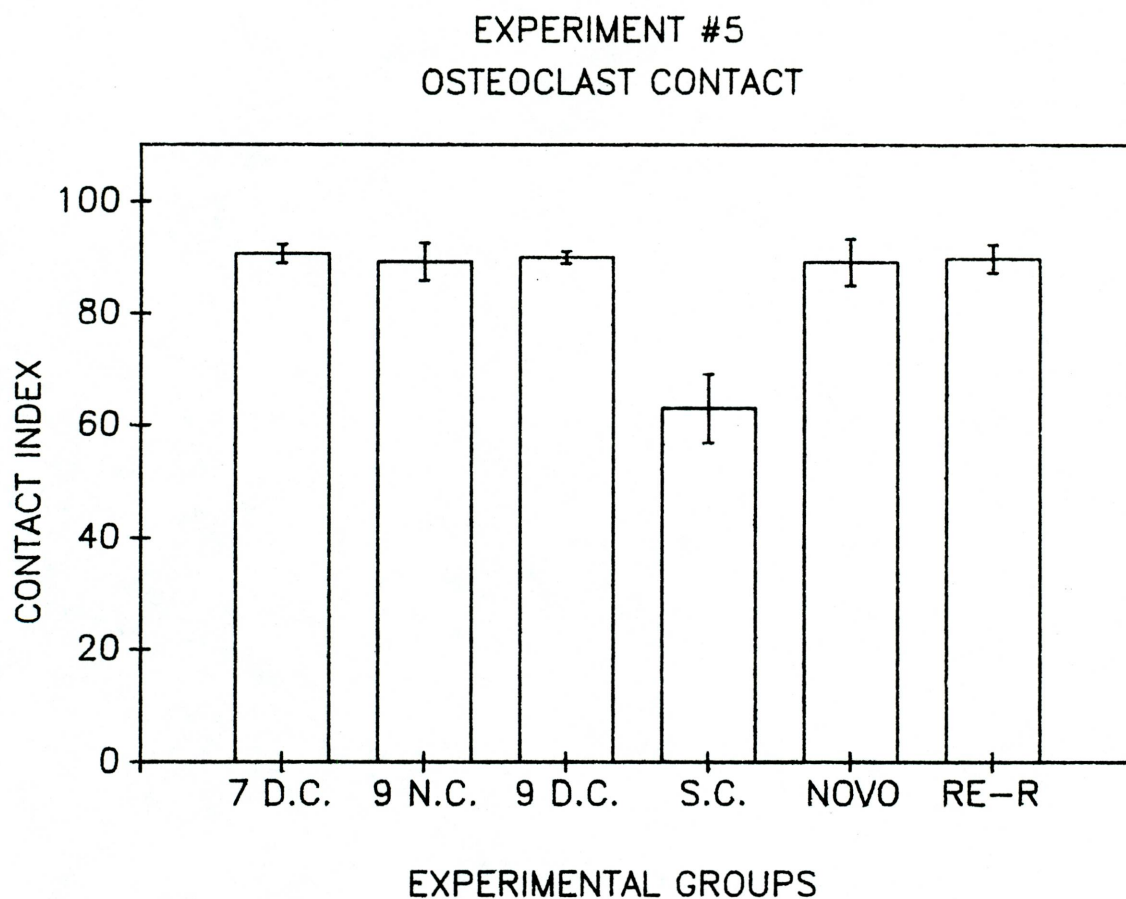


FIGURE 16: Osteoclast Contact Index, means \pm SEM. **Experimental groups:**
7 D.C. = 7-day deficient control, 9 N.C. = 9-day normal control,
9 D.C. = 9-day deficient control, S.C. = supplemented control,
NOVO = *de novo* recruitment, RE-R = re-recruitment. $n = 6$ for all groups.

EXPERIMENT #5
OSTEOCLAST SEPARATION

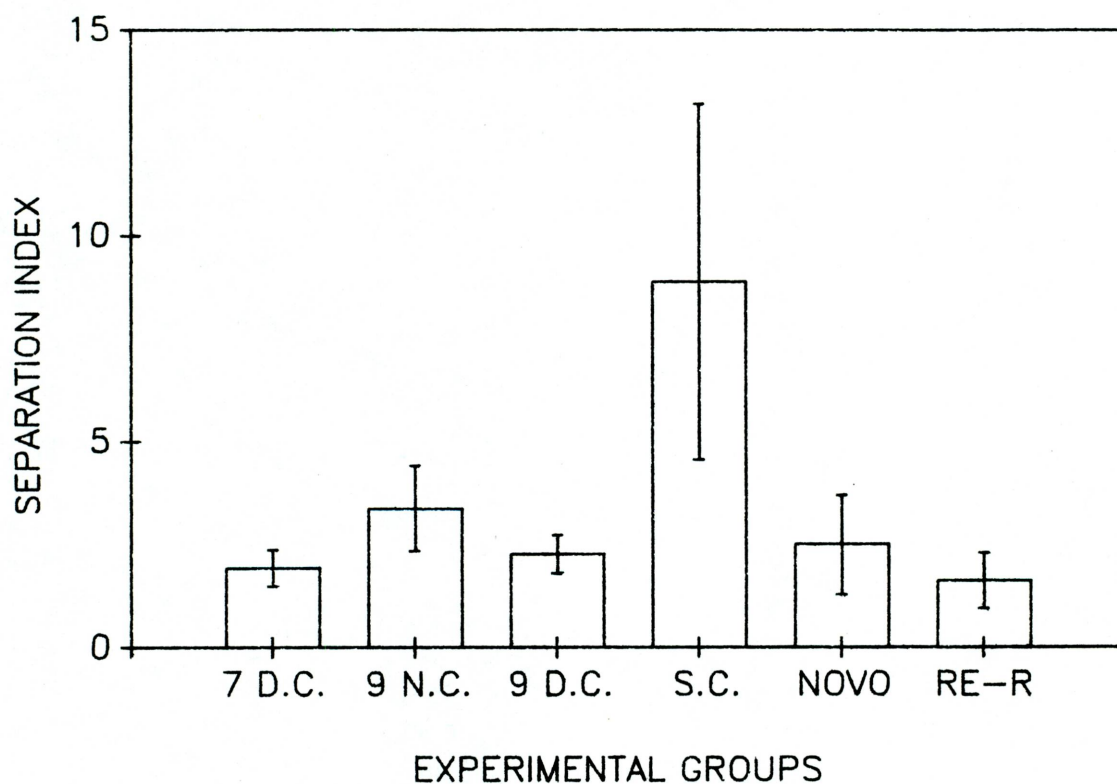


FIGURE 17: Osteoclast Separation Index, means \pm SEM. **Experimental groups:**
7 D.C. = 7-day deficient control, 9 N.C. = 9-day normal control,
9 D.C. = 9-day deficient control, S.C. = supplemented control,
NOVO = *de novo* recruitment, RE-R = re-recruitment. $n = 6$ for all groups.

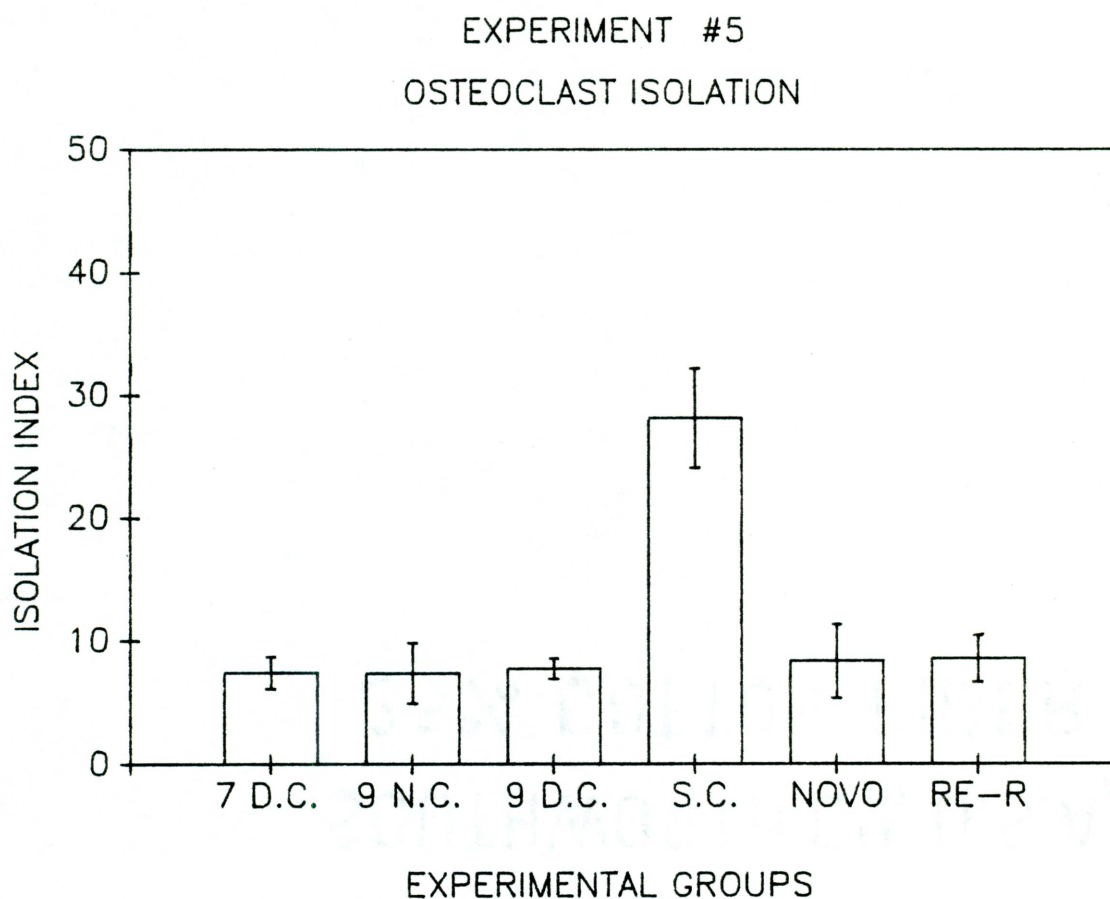


FIGURE 18: Osteoclast Isolation Index, means \pm SEM. **Experimental groups:**
7 D.C. = 7-day deficient control, 9 N.C. = 9-day normal control,
9 D.C. = 9-day deficient control, S.C. = supplemented control,
NOVO = *de novo* recruitment, RE-R = re-recruitment. $n = 6$ for all groups.

To measure the profile width, evenly spaced lines were placed across each osteoclast profile perpendicular to the bone surface, and the distance between the two points where the line intersected the osteoclast cell boundary was measured. The two deficient control groups showed significantly greater osteoclast profile widths than all the other groups except the re-recruitment group (Figure 19, page 78), and while there was not a significant difference between the osteoclast profile width of the *de novo* recruitment and the re-recruitment groups, these two groups had greater profile widths than either the normal control or the supplemented control groups.

The interesting comparison here is between the *de novo* recruitment group and the re-recruitment group. If re-recruitment does in fact take place following two days of calcium-induced inhibition, one would expect the parameters indicating increased osteoclastic activity, i.e., OCAF, RBI and CI, to be greater in the re-recruitment group than in the *de novo* group. Such is not the case here. The OCAF is actually greater (though not statistically) in the *de novo* group, while the re-recruitment group shows much greater osteoblast activity and a lower resting cell fraction than any of the other groups. What seems to be happening here is that, as was shown in a previous study, the osteoblasts are turned on only after two days of supplementation. There is evidently a sort of rebound phenomenon - the osteoblasts become "hyperactive" at the expense of the resting cell fraction. While the OCAF is greater in the re-recruitment group than in the normal and supplemented controls, it does not yet approach deficient control levels, and hasn't even quite caught up with the *de novo* recruitment level.

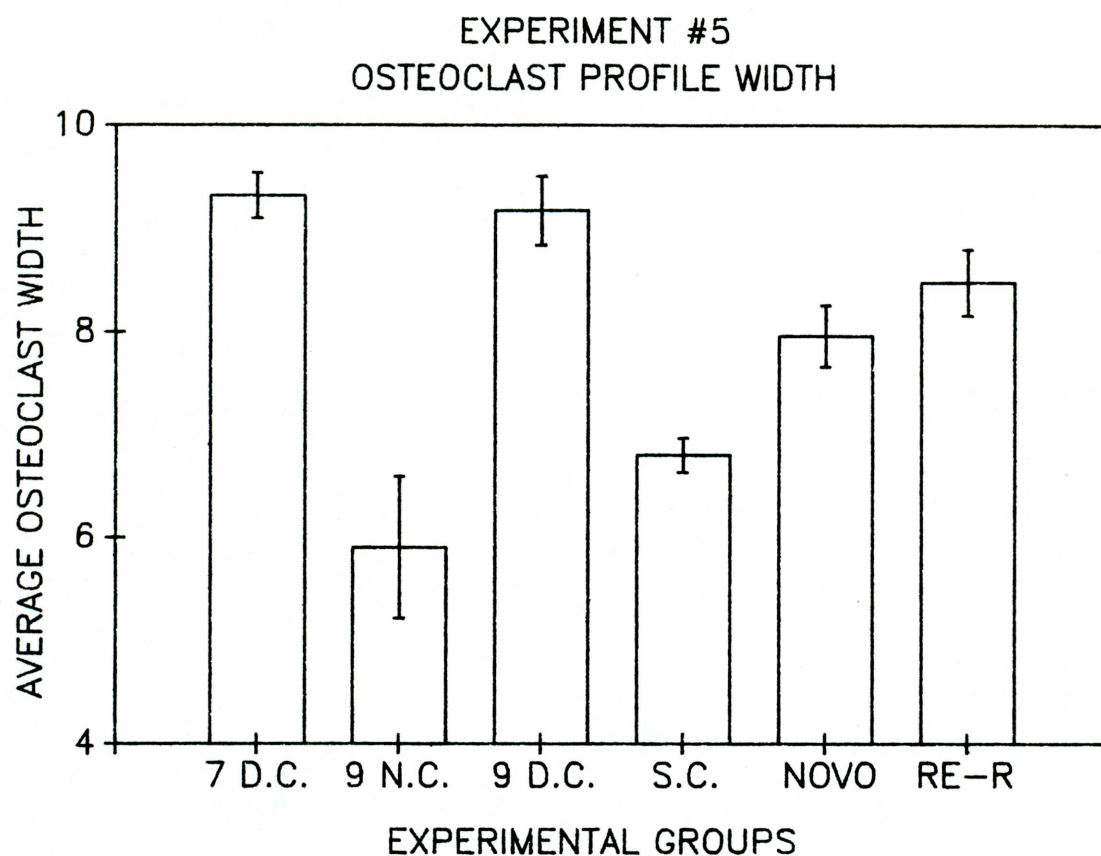


FIGURE 19: Osteoclast Profile Width, means \pm SEM. Experimental groups:
7 D.C. = 7-day deficient control, 9 N.C. = 9-day normal control,
9 D.C. = 9-day deficient control, S.C. = supplemented control,
NOVO = *de novo* recruitment, RE-R = re-recruitment. $n = 6$ for all groups.

The supplemented normal control group behaves as would be predicted, with osteoblast and osteoclast levels returning to normal control levels after two days calcium supplementation. While the *de novo* group differs significantly from the re-recruitment group in the OBAF and RAF, the difference between the *de novo* and the normal control groups in the OCAF only approaches significance, indicating that 2 days is not really enough to see the effects of calcium deficiency; the OCAFs for the two deficient controls are significantly greater than the OCAF for the *de novo* recruitment group, and the osteoclast profile widths for these two groups are also significantly greater than in the *de novo* group. Though the profile width in the re-recruitment group is not statistically different from the *de novo* group, it does show a tendency to approach deficient levels, suggesting that while the OCAF has decreased, those osteoclasts that remain still show a relatively high degree of activation.

DISCUSSION

As was previously discussed, bone metabolism is partly under the control of hormones. In addition to PTH, CT, D₃ and estrogen, growth hormone (GH) influences bone cells. The actions of growth hormone are mediated by a class of compounds formerly called somatomedins, and now known as insulin-like growth factors (IGF), because of their similarity in structure and function to insulin. The IGFs are carried in the blood by several carrier proteins, called IGF binding proteins (IGFBPs).

Fasting has been shown to affect the GH/IGF axis. Several studies have shown that fasting decreases serum IGF-I (somatomedin-C) levels (Phillips and Young 1976; Clemmons et al. 1981; Merimee, Zapf and Froesch 1982; Isley, Underwood and Clemmons 1983), as well as extractable hepatic IGF-I (Goldstein, Harp and Phillips 1991). This fall in serum IGF-I levels has been shown to correlate with a decrease in the number of hepatic GH binding sites (Maes, Underwood, and Ketelslegers 1983), and with decreases in T3 and insulin production (Caufriez et al. 1984). The decrease in IGF-I appears to be due to a decrease in IGF-I mRNA levels with fasting (Emler and Schalch 1987; Lowe et al. 1989; Goldstein, Harp and Phillips 1991). Fasting has also been shown to decrease IGFBP levels (McCusker et al. 1991).

GH has been shown to affect both bone formation and bone resorption. Brixen et al. (1990) injected normal human volunteers twice daily for seven days with recombinant human GH, and measured resultant changes in biochemical markers for bone formation and resorption. They found that at first both

formation and resorption increased as measured by changes in urinary hydroxyproline/creatinine and calcium/creatinine ratios, and by serum bone Gla protein and alkaline phosphatase levels. Within a few weeks, however, resorption parameters decreased, but formation parameters remained elevated for long periods of time. The authors concluded that GH stimulated osteoblast activity and bone remodeling. Similar results were reported by Schlemmer et al. (1991). They gave daily injections of synthetic human GH to GH-deficient patients over a period of four months. This caused rises in fasting urinary excretion of pyridinoline and deoxypyridinoline, two newly discovered markers of bone resorption. However, there was also a corresponding increase in plasma bone Gla protein, which is a marker of bone formation. These authors also concluded that GH injections increase bone remodeling. Hock and Fonesca (1990) showed that PTH produced anabolic effects in rat bones only if GH was present. After injecting PTH into hypophysectomized (HX) and sham-operated male rats daily for 12 days, they found that in the sham animals there was an increase in bone calcium, bone dry weight and hydroxyproline, but in HX rats, these anabolic parameters were absent. Injecting GH with PTH restored the anabolic effects produced by PTH. IGF was also shown to stimulate bone formation in calvarial cultures (Canalis, 1980; Hock, Centrella and Canalis 1988; Canalis et al. 1989; Spencer et al. 1991). Linkhart and Mohan (1989) reported that PTH stimulated calvarial osteoblasts in culture to release IGF-I and II, which in turn stimulated osteoblastic proliferation. They concluded that IGF participated in the coupling of formation and resorption.

Spencer et al. (1991) reported that infusion of IGF-I into the right hindlimb of ambulatory rats increased bone formation, but decreased the number of osteoclasts, leading them to conclude that IGF-I is purely anabolic in its actions. However, Sloodweg et al. (1992) reported that adding IGF-I to fetal mouse radii/ulnae cultures containing mature osteoclasts did not produce a response, but when IGF-I was added to metacarpal/metatarsal cultures containing osteoclast precursor cells, the number of osteoclasts increased, and bone resorption also increased. They concluded that IGF-I has a stimulatory effect on osteoclast precursors but not on mature osteoclasts.

These studies could provide clues about how fasting brings about the changes that were observed in these experiments. The fasting experiments in this study have provided several pieces of information. First, it was shown that short-term fasting can reverse osteoclast recession induced by calcium supplementation in calcium-deficient rats. Second, it was found that when normal rats are fasted for 48 hours, the OBAF decreases dramatically concomitantly with an increase in the RAF. This apparent reduction in osteoblast activity might be explained in part on the basis of a reduction in GH and IGF activity, since some of the above studies show that GH and IGF-I stimulate osteoblastic activity and bone formation. The reduction in IGF-I might also explain the reason why, in spite of the presumed decrease in blood calcium levels with fasting, the OCAF did not increase in the fasting animals, since as Sloodweg et al. (1992) demonstrated, IGF-I has a stimulatory effect on osteoclast precursors. Additionally, osteoblasts are

known to secrete factors such as IL-1 and IL-6 that stimulate osteoclast activity. A reduction in osteoblast activity might also contribute in this way to a reduction in the OCAF.

The results of this study have confirmed that while a calcium-deficient diet is capable of recruiting large numbers of osteoclasts to rat tibial diaphyses, feeding deficient animals a calcium-containing diet for 12-48 hours causes osteoclasts to recede from bone. It was shown that osteoclast recession is a graded process, and that a 12-hour fasting period can reverse the effects of 12 hours of calcium supplementation on osteoclast morphology. The fast presumably lowers plasma calcium levels, which would cause a decrease in calcitonin levels and an increase in levels of PTH. Either of these factors, or a combination of both, could account for the reactivation of inhibited osteoclasts due to fasting.

While 48 hours of calcium deficiency in normal rats has been shown to be sufficient to significantly increase the osteoclast area fraction of the tibial endosteum at the expense of the RAF, 48 hours of fasting in normal rats increases the resting area fraction of the endosteum while decreasing the osteoblast area fraction. Thus, short-term fasting operates by reducing bone formation rather than by increasing bone resorption. It must be noted that the action of fasting on bone is probably much more complex than simply causing a reduction in blood calcium. As was pointed out earlier, fasting produces many effects in the body, including changes in carbohydrate, lipid and protein metabolism. The effects of these changes on bone cells is not known.

Finally, this study has shown that if inhibited osteoclasts can be re-recruited to bone, it apparently takes longer than 48 hours of calcium deficiency to bring the osteoclasts back to pre-inhibition levels of activity.

APPENDIX A

CUSTOM CALCIUM FREE DIET WITH 0.4% PHOSPHORUS

Vitamin free casein	2230 gm
Alphacel non-nutritive bulk	1120 gm
Corn oil	500 gm
Sucrose	1731 gm
Corn starch	4065 gm
Vitamin diet fortification mixture:	
Monopotassium phosphate	88 gm
Monosodium phosphate	77 gm
Copper sulfate $5\text{H}_2\text{O}$	0.300 gm
Ferrous sulfate $7\text{H}_2\text{O}$	10.800 gm
Magnesium sulfate $7\text{H}_2\text{O}$	40.600 gm
Manganese Sulfate H_2O	1.800 gm
Sodium chloride	80 gm
Potassium chloride	48 gm
Potassium iodide	0.100 gm
Sodium selenite	0.010 gm
Zinc oxide	0.750 gm
Chromium potassium sulfate $12\text{H}_2\text{O}$	0.220 gm
Ammonium molybdate $4\text{H}_2\text{O}$	0.020 gm

CUSTOM NORMAL DIET WITH 0.7% CALCIUM AND 0.7% PHOSPHORUS

Vitamin free casein	2230 gm
Alphacel non-nutritive bulk	1120 gm
Corn oil	500 gm
Corn starch	4065 gm
Sucrose	1500 gm
Vitamin diet fortification mixture:	
Monopotassium phosphate	154.000 gm
Monosodium phosphate	135.000 gm
Calcium carbonate	175.000 gm
Copper sulfate $5\text{H}_2\text{O}$	0.300 gm
Ferrous sulfate $7\text{H}_2\text{O}$	10.800 gm
Magnesium sulfate $7\text{H}_2\text{O}$	40.600 gm
Manganese sulfate H_2O	1.800 gm
Sodium chloride	50.000 gm
Potassium chloride	10.000 gm
Potassium iodide	0.100 gm
Sodium selenite	0.010 gm
Zinc oxide	0.750 gm
Chromium potassium sulfate $12\text{H}_2\text{O}$	0.220 gm
Ammonium molybdate $4\text{H}_2\text{O}$	0.020 gm

APPENDIX B

EXPERIMENT #1: NORMAL CONTROL

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
1 5	38.7	55.9	5.4	11.1	95.8	0.3	3.9	0.3
1 7	41.4	47.6	11.0	23.6	91.3	0.0	8.7	0.0
1 9	41.8	44.7	13.5	15.7	86.3	8.9	4.8	0.4
2 7	38.9	56.9	4.3	6.3	94.5	0.9	4.6	0.1
2 8	33.7	57.9	8.4	17.0	97.1	1.7	1.2	0.0
3 3	25.3	56.4	18.4	18.8	93.8	0.0	6.2	0.0
MEAN	36.6	53.2	10.2	15.4	93.1	2.0	4.9	0.1
SD	5.7	5.1	4.8	5.5	3.5	3.2	2.3	0.2

EXPERIMENT #1: DEFICIENT CONTROL

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
5	21.6	51.2	27.2	19.7	80.9	11.4	7.7	0.7
1 2	18.8	58.4	22.9	17.2	88.0	4.3	7.7	1.6
1 8	9.5	49.1	41.4	24.0	88.7	2.3	9.0	0.7
2 3	22.1	52.2	25.8	24.6	88.0	3.5	8.5	0.0
3 1	9.4	41.7	48.9	16.0	94.0	0.2	5.8	0.1
3 8	32.5	40.0	27.4	13.5	87.8	3.6	8.6	0.7
MEAN	19.0	48.8	32.3	19.2	87.9	4.2	7.9	0.6
SD	8.0	6.3	9.5	4.1	3.8	3.3	1.0	0.5

EXPERIMENT #1: 3 - HOUR SUPPLEMENTED

Animal #	OBAF	RAF	OCAF	RRI	CI	SI	II	REM
1	23.9	56.9	19.2	25.4	89.8	5.8	4.4	0.2
8	D	E	C	E	A	S	E	D
9	11.2	63.1	25.7	19.6	86.9	4.7	8.4	0.0
2 5	29.9	56.8	13.3	27.4	82.8	5.9	11.2	0.0
3 2	18.5	50.1	31.4	27.8	90.5	5.0	4.6	0.0
4 1	42.1	38.7	19.2	12.0	90.4	1.1	8.5	0.4
MEAN	25.1	53.1	21.8	22.4	88.1	4.5	7.4	0.1
SD	11.7	9.3	6.9	6.7	3.3	2.0	2.9	0.2

EXPERIMENT #1: 6 - HOUR SUPPLEMENTED

Animal #	OBAF	RAF	OCAF	RRI	CI	SI	II	REM
1 6	9.9	58.8	31.3	18.2	87.3	5.4	7.2	1.2
2 0	46.7	31.5	21.8	19.7	94.6	1.2	4.3	0.3
2 1	30.9	41.4	27.7	23.0	84.3	5.9	9.8	0.0
2 2	29.3	45.0	25.7	26.4	94.1	0.3	5.6	0.3
3 4	15.8	36.1	48.1	12.9	94.2	0.7	5.1	0.0
4 2	30.4	42.1	27.5	13.8	84.8	5.6	9.6	0.1
MEAN	27.2	42.5	30.4	19.0	89.9	3.2	6.9	0.3
SD	12.9	9.3	9.2	5.2	4.9	2.7	2.3	0.5

EXPERIMENT #1: 9 - HOUR SUPPLEMENTED

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
2	13.4	47.7	38.9	1.7	74.2	16.2	9.6	2.0
3	24.4	51.2	24.4	8.9	85.2	8.5	6.3	0.3
6	15.1	58.4	26.4	9.0	86.6	7.5	5.9	0.9
2 4	24.8	52.1	23.2	18.7	92.4	0.7	6.9	0.0
2 9	27.0	57.8	15.2	18.3	91.2	4.1	4.7	0.3
3 0	28.8	48.9	22.3	14.9	90.4	4.5	5.0	0.3
MEAN	22.3	52.7	25.1	11.9	86.7	6.9	6.4	0.6
SD	5.9	4.1	7.1	6.0	6.1	4.9	1.6	0.7

EXPERIMENT #1: 12 - HOUR SUPPLEMENTED

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
7	12.0	67.6	20.3	16.5	91.3	5.4	3.3	1.0
1 0	32.8	46.4	20.8	23.5	93.5	4.3	2.2	0.0
1 1	18.2	68.0	13.8	16.8	91.4	3.6	5.0	0.1
2 6	7.8	67.9	24.3	18.3	91.0	2.2	6.7	0.1
3 6	24.7	47.3	28.0	17.7	94.5	2.7	2.8	0.0
3 7	37.2	42.2	20.6	4.9	91.5	0.9	7.6	0.5
MEAN	22.1	56.6	21.3	16.3	92.2	3.2	4.6	0.3
SD	11.6	12.5	4.7	6.1	1.4	1.6	2.2	0.4

EXPERIMENT #1: TOTAL SUPPLEMENTED

Animal #	OBAF	BAF	OCAF	RBI	CI	SI	II	REM
4	15.3	57.7	27.1	12.7	95.5	1.5	3.0	0.0
1 3	27.9	52.2	20.0	19.4	73.0	17.6	9.4	0.3
1 4	17.8	62.0	20.2	21.0	91.0	3.1	5.9	0.0
3 5	16.4	53.3	30.3	11.1	94.4	0.7	4.8	0.1
3 9	20.2	54.7	25.2	1.5	79.4	3.8	16.8	0.3
4 0	45.8	31.3	22.9	11.3	89.4	2.5	8.2	0.0
MEAN	23.1	51.8	25.1	11.5	89.9	2.3	7.7	0.1
SD	11.5	10.7	3.5	6.2	5.7	1.1	4.8	0.1

EXPERIMENT #2: NORMAL CONTROL

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
3	22.6	70.7	6.6	4.5	95.0	0.0	5.0	0.0
4	29.7	65.9	4.4	38.5	94.2	0.0	5.8	0.0
6	60.4	36.6	3.0	27.2	78.8	0.0	21.2	0.0
17	42.6	52.0	5.8	5.7	77.0	7.4	15.5	0.0
19	38.3	49.5	12.8	24.4	85.2	4.5	10.3	0.0
20	42.7	50.3	8.5	2.5	63.9	17.2	18.9	0.0
MEAN	39.4	54.2	6.9	17.1	82.4	4.9	12.8	0.0
SD	13.0	12.3	3.5	14.9	11.7	6.8	6.8	0.0

EXPERIMENT #2: DEFICIENT CONTROL

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
11	8.3	55.7	37.0	19.5	87.1	2.5	10.4	0.2
16	29.0	47.9	24.0	28.7	86.1	3.5	10.4	0.0
18	31.3	56.5	13.5	12.0	78.3	10.0	11.7	0.0
22	16.0	42.3	43.7	19.3	84.1	4.7	11.2	0.0
23	24.9	47.3	27.8	31.1	89.7	0.1	10.2	0.0
24	35.1	41.2	24.5	29.6	81.6	3.4	15.0	0.0
MEAN	24.1	48.5	28.4	23.4	84.5	4.0	11.5	0.0
SD	10.1	6.5	10.6	7.6	4.1	3.3	1.8	0.1

EXPERIMENT #2: FASTING SUPPLEMENTED

Animal #	OBAF	BAF	OCAF	RBI	CI	SI	II	REM
1	20.5	62.0	18.8	23.7	86.8	6.7	6.6	0.0
5	3.7	74.0	24.1	13.4	79.3	7.8	12.9	0.0
7	19.2	51.5	30.8	21.8	86.3	4.9	8.8	0.0
10	9.7	73.2	17.8	13.3	79.7	4.1	16.2	0.0
13	24.8	60.6	15.3	15.9	83.9	4.4	11.7	0.0
15	30.2	51.0	18.9	24.9	89.5	0.5	10.0	0.0
MEAN	18.0	62.1	21.0	18.8	84.3	4.7	11.0	0.0
SD	9.8	10.0	5.6	5.3	4.1	2.5	3.4	0.0

EXPERIMENT #2: NON-FASTING SUPPLEMENTED

Animal #	OBAF	BAF	OCAF	RBI	CI	SI	II	REM
2	26.0	59.0	17.6	11.7	68.4	14.7	16.9	0.4
8	29.8	62.1	9.5	8.3	69.6	15.1	15.3	0.0
9	19.7	64.0	17.6	4.4	68.2	7.6	24.2	0.0
12	29.8	56.0	16.7	9.6	60.1	15.1	24.9	0.0
14	18.5	73.1	10.7	9.3	55.3	21.1	23.6	0.0
21	27.9	56.3	18.0	7.6	60.1	12.4	27.5	0.1
MEAN	25.3	61.8	15.0	8.5	63.6	14.3	22.1	0.1
SD	5.0	6.4	3.9	2.4	5.9	4.4	4.8	0.2

EXPERIMENT #3: RESTRICTED SUPPLEMENTED

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
5	23	61.2	16.2	16.1	85.3	2.4	12.2	0.0
8	34.7	51.3	14.8	16.4	81	5.3	13.7	0.0
9	18.3	55.3	27.1	16.7	83.4	2.6	14	0.0
1 3	20.1	63.1	17.2	7.8	70	1.8	28.3	0.0
1 5	21.8	57.7	20.7	6.9	76.1	1.0	22.9	0.0
1 7	26.9	51.6	22.6	18.1	77.4	4.9	17.7	0.0
MEAN	24.1	56.7	19.8	13.7	78.9	3.0	18.1	0.0
SD	5.9	4.9	4.6	4.9	5.6	1.7	6.3	0.0

EXPERIMENT #3: 12-HOUR AD LIB

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
1	21.0	70.3	9.4	1.9	70.2	7.5	22.3	0.0
2	7.3	77.3	15.9	1.5	66.8	3.9	29.3	0.0
3	13.8	74.1	12.9	3.5	62.1	5.7	32.2	0.0
6	31.0	58.0	11.5	4.2	62.5	5.0	32.6	0.0
7	37.9	55.1	7.0	6.1	76.2	0.7	23.1	0.0
1 1	16.1	70.2	14.2	2.6	65.6	3.3	31.0	0.0
MEAN	21.2	67.5	11.8	3.3	67.2	4.4	28.4	0.0
SD	11.4	8.9	3.2	1.7	5.3	2.3	4.6	0.0

EXPERIMENT #3: 24-HOUR AD LIB

Animal #	OBAF	RAF	OCAF	RRI	CI	SI	II	REM
4	24.9	66.6	8.5	17.2	77.7	0.2	22.1	0.0
10	30.4	62.0	8.1	6.2	69.0	5.9	25.1	0.0
12	35.0	60.8	4.5	0.0	47.9	7.4	44.7	0.0
14	37.9	55.0	7.1	12.4	75.6	0.0	24.4	0.0
16	29.5	61.6	8.9	5.2	83.0	0.8	16.1	0.0
18	36.4	57.5	6.4	10.0	73.1	3.2	23.7	0.0
MEAN	32.4	60.6	7.3	8.5	71.1	2.9	26.0	0.0
SD	4.9	4.0	1.6	6.0	12.3	3.1	9.7	0.0

EXPERIMENT #4: 24 - HOUR FASTED

Animal #	ORAF	RAF	OCAF	RRI	CI	SI	II	REM
4	24.5	61.5	14.1	16.9	90.4	1.0	8.6	0.0
14	74.8	22.0	3.3	39.1	96.2	0.0	3.8	0.0
15	29.3	54.8	16.0	33.4	96.2	0.7	3.1	0.0
16	51.2	35.8	13.1	35.1	94.3	0.5	5.2	0.0
20	29.9	64.2	6.0	18.3	94.1	0.0	5.9	0.0
22	51.3	36.5	12.2	33.4	89.8	0.0	10.2	0.0
MEAN	43.5	45.8	10.8	29.4	93.5	0.4	6.1	0.0
SD	19.2	16.8	5.0	9.4	2.8	0.4	2.8	0.0

EXPERIMENT #4: 48 - HOUR FASTED

Animal #	ORAF	RAF	OCAF	RRI	CI	SI	II	REM
1	13.4	73.5	13.2	49.2	93.7	0.3	6.0	0.0
8	11.5	74.5	14.1	23.3	96.2	0.8	3.1	0.0
10	32.2	61.9	6.0	19.0	92.0	1.8	6.2	0.0
11	22.1	68.2	9.7	20.8	95.7	0.0	4.3	0.0
19	8.8	89.3	1.9	0.0	82.2	3.5	14.3	0.0
23	18.1	75.0	6.9	38.3	96.0	0.0	4.0	0.0
MEAN	17.7	73.7	8.6	25.1	92.6	1.1	6.3	0.0
SD	8.6	9.1	4.6	17.0	5.4	1.4	4.1	0.0

EXPERIMENT #4: 24 - HOUR DEFICIENT

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
2	15.8	64.0	20.2	32.7	97.6	0.2	2.2	0.0
7	53.2	38.7	8.8	6.5	84.6	8.0	7.5	0.0
9	50.4	41.2	8.4	22.1	92.1	0.0	7.9	0.0
13	72.7	24.9	2.4	24.3	98.2	0.0	1.8	0.0
17	57.0	37.3	5.7	9.6	97.8	0.8	1.3	0.0
18	56.7	28.3	15.0	25.6	91.0	0.0	9.0	0.0
MEAN	51.0	39.1	10.1	20.1	93.6	1.5	5.0	0.0
SD	18.9	13.8	6.5	10.1	5.4	3.2	3.5	0.0

EXPERIMENT #4: 48 - HOUR DEFICIENT

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM
3	43.7	43.5	13.0	28.5	89.2	1.2	9.7	0.0
5	43.4	32.2	24.6	5.1	91.5	0.8	7.7	0.0
6	48.6	39.5	13.6	3.9	74.6	12.1	13.4	0.0
12	32.9	57.9	9.2	10.1	99.5	0.0	0.5	0.0
21	23.9	47.4	28.7	26.2	95.9	0.0	4.1	0.0
24	40.9	44.6	14.5	26.6	96.3	0.0	3.7	0.0
MEAN	38.9	44.2	17.3	16.7	91.2	2.4	6.5	0.0
SD	9.0	8.5	7.6	11.6	8.9	4.8	4.7	0.0

EXPERIMENT #5: 7 - DAY DEFICIENT CONTROL

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM	PROF
6	21.6	56.3	22.2	18.1	90.3	1.8	8.0	0.0	ND
10	39.3	38.0	22.7	19.4	84.9	3.6	11.5	0.0	9.5
27	32.9	40.3	26.8	25.8	89.0	1.6	9.4	0.1	8.6
33	45.9	36.1	18.1	33.3	93.9	1.2	4.9	0.0	9.9
34	33.8	34.1	32.2	32.3	96.6	0.6	2.8	0.0	9.5
35	43.7	39.6	16.7	35.6	89.4	2.7	7.9	0.0	9.1
MEAN	36.2	40.7	23.1	27.4	90.7	1.9	7.4	0.0	9.3
SD	8.8	8.0	5.7	7.5	4.1	1.1	3.1	0.0	0.5

EXPERIMENT #5: 9 - DAY NORMAL CONTROL

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM	PROF
1	51.2	43.7	5.1	10.6	84.4	6.1	9.5	0.0	ND
4	32.5	65.9	1.6	9.3	98.3	1.7	0.0	0.0	ND
7	60.4	30.4	9.2	33.1	78.6	6.1	15.3	0.0	ND
17	39.0	54.2	6.8	9.5	91.5	1.8	6.7	0.0	7.1
19	73.8	24.9	1.3	11.7	84.0	4.5	11.5	0.0	4.7
22	35.1	56.4	8.5	20.0	98.8	0.0	1.2	0.0	5.9
MEAN	48.7	45.9	5.4	15.7	89.2	3.4	7.4	0.0	5.9
SD	16.2	15.9	3.4	9.4	8.3	2.6	6.0	0.0	1.2

EXPERIMENT #5: 9 - DAY DEFICIENT CONTROL

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM	PROF
5	46.0	40.9	13.0	39.0	93.2	1.2	5.7	0.0	ND
9	39.6	37.4	22.9	23.7	91.1	1.8	7.1	0.0	ND
21	27.8	52.3	19.9	20.7	90.0	1.6	8.4	0.0	8.5
23	43.0	35.4	21.7	29.5	85.8	4.2	10.0	0.0	9.1
26	24.4	46.2	29.5	21.0	88.5	1.7	9.8	0.0	9.0
28	46.8	37.7	15.5	23.9	91.7	3.0	5.3	0.0	10.1
MEAN	37.9	41.7	20.3	26.3	90.1	2.3	7.7	0.0	9.2
SD	9.6	6.4	6.3	7.0	2.6	1.1	2.0	0.0	0.5

EXPERIMENT #5: SUPPLEMENTED CONTROL

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM	PROF
3	63.1	33.9	3.0	0.0	42.2	30.1	27.7	0.0	ND
14	50.8	42.7	6.6	4.7	63.8	6.6	29.7	0.0	7.1
15	53.2	42.0	4.8	7.4	55.9	3.7	40.3	0.0	6.9
20	42.0	54.8	3.1	10.8	87.8	1.2	11.0	0.0	6.7
29	26.6	70.4	3.0	0.0	60.2	5.3	34.5	0.0	7.1
30	44.3	53.6	2.1	0.0	68.2	6.4	25.5	0.0	6.2
MEAN	46.7	49.6	3.8	3.8	63.0	8.9	28.1	0.0	6.8
SD	12.3	12.9	1.6	4.6	15.1	10.6	9.9	0.0	0.4

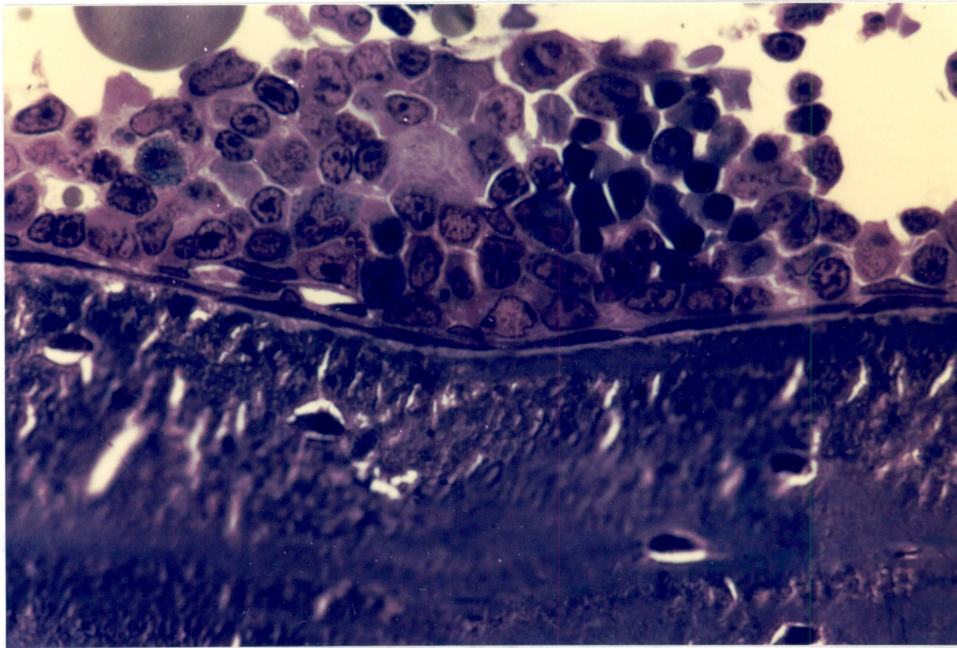
EXPERIMENT #5: DE NOVO RECRUITMENT

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM	PROF
2	45.3	38.1	16.7	34.9	82.2	4.0	13.8	0.0	ND
11	34.6	42.4	23.0	25.5	90.1	0.7	9.2	0.0	8.4
12	62.8	30.0	7.2	13.6	72.2	7.9	19.9	0.0	6.9
16	42.8	53.6	3.6	7.3	96.5	0.7	2.8	0.0	8.3
31	36.2	51.6	12.2	27.9	97.9	0.6	1.5	0.0	8.5
32	21.6	73.9	4.5	26.0	96.2	1.0	2.9	0.0	7.7
MEAN	40.6	48.3	11.2	22.5	89.2	2.5	8.4	0.0	8.0
SD	13.7	15.3	7.6	10.1	10.2	3.0	7.4	0.0	0.7

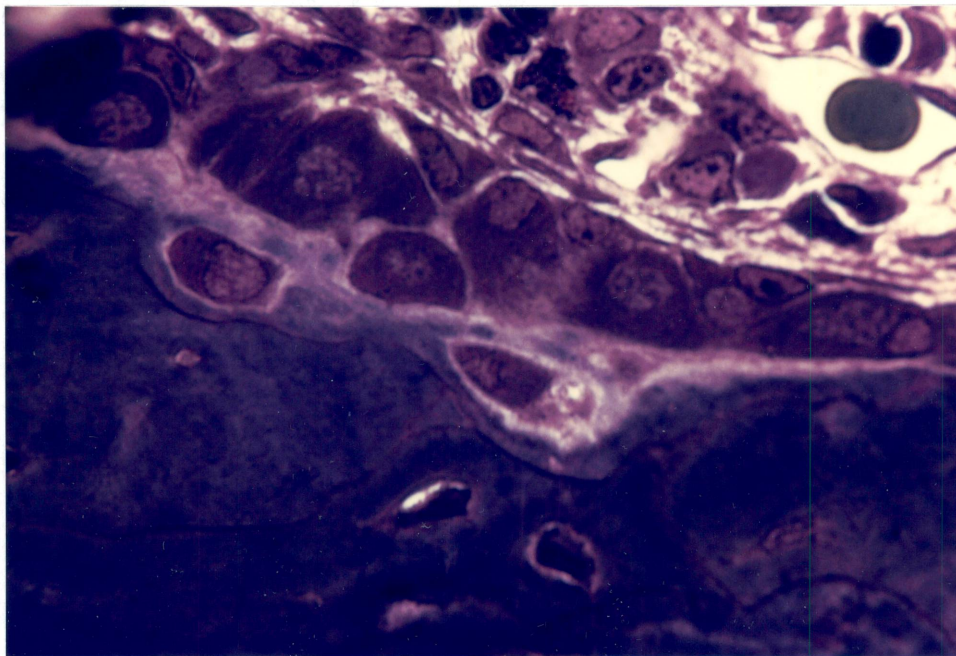
EXPERIMENT #5: RE-RECRUITMENT

Animal #	OBAF	RAF	OCAF	RBI	CI	SI	II	REM	PROF
8	71.7	18.4	10.0	36.5	86.7	2.1	11.1	0.0	ND
13	64.9	23.6	11.6	31.3	87.1	1.6	11.2	0.0	9.0
18	64.2	30.0	5.8	23.9	91.2	0.6	8.1	0.0	8.1
24	58.5	31.3	10.2	27.6	81.4	4.6	14.0	0.0	9.0
25	80.7	15.4	4.0	31.6	99.1	0.0	0.9	0.0	8.9
36	70.7	22.1	7.2	23.0	93.2	0.8	6.0	0.0	7.4
MEAN	68.5	23.5	8.1	29.0	89.8	2.8	8.6	0.0	8.5
SD	7.7	6.3	2.9	5.2	6.1	3.0	4.7	0.0	0.7

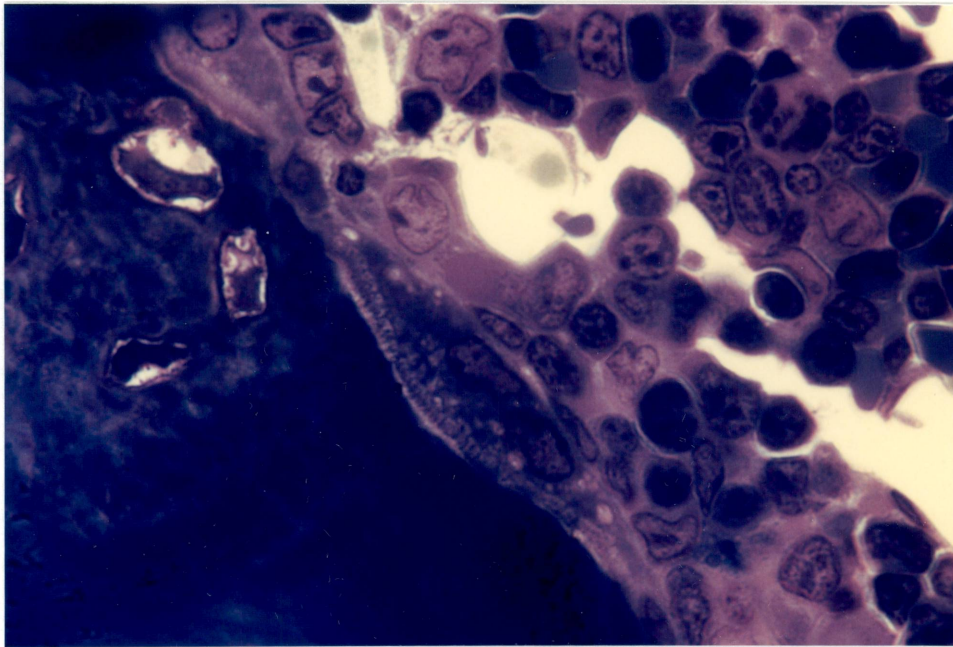
APPENDIX C



RESTING CELLS - layer of very flat cells covering the bone



OSTEOBLASTS - the layer of large cells covering the bone



OSTEOCLAST - large, multinucleated cell lying on the bone surface. Note the ruffled border adjacent to the bone.

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